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Epidemiological studies suggest that dietary folate intake and blood levels of folate are inversely related to breast cancer risk. Because only few modifiable risk factors for breast cancer exist, the role of folate in modifying breast cancer risk merits further consideration. Folate is an ideal agent for chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects, and possesses biologically plausible mechanisms for cancer prevention. However, folate appears to possess dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention. Folate deficiency has an inhibitory, whereas folate supplementation has a promoting, effect on progression of established neoplasms. By contrast, folate deficiency in normal tissues predisposes them to neoplastic transformation, and modest levels of folate supplementation suppress, whereas supraphygiologic doses enhance, the development of tumors in normal tissues. Therefore, the potential effect of folate chemoprevention needs to be clearly established in appropriate animal models before folate supplementation can be considered in humans. Given these considerations, this proposal investigates the effects of dietary folate deficiency and supplementation on mammary tumorigenesis and potential molecular and cellular mechanisms by which folate modulates mammary tumorigenesis in the well established carcinogen rat model of breast cancer.

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## INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer deaths in women in the United States (*1*). Genetic predisposition (*2-4*) and hormonal/reproductive factors (*5-7*), two important determinants of breast cancer risk, are not readily modifiable. Therefore, much effort has been directed towards identifying potentially modifiable dietary and lifestyle factors that would lead to the prevention of breast cancer. Although epidemiological and animal studies have suggested that dietary factors, such as fat, fiber, vegetables and fruits, antioxidants and alcohol, may influence breast cancer risk, effects of these factors on breast cancer risk are inconsistent and contradictory (*1*). As such, prevention of breast cancer through dietary modifications remains an elusive and challenging task.

Folate, a water-soluble B-vitamin and important co-factor in one-carbon metabolism, has recently been identified as an important nutritional factor that may modulate carcinogenesis (*8-10*). The role of folate in carcinogenesis has been best studied for colorectal cancer (*8-10*). The majority of over 25 published epidemiological studies indicate that dietary folate intake and blood folate levels are inversely associated with colorectal cancer risk (*8-10*). Collectively, these studies suggest an ~40% reduction in the risk of colorectal neoplasms in subjects with highest dietary folate intake compared with those with the lowest intake (*8-10*). These studies also suggest that a modest reduction in folate status is sufficient to enhance colorectal cancer risk (*8-10*). Animal studies have also been generally supportive of a causal relationship between folate depletion and colorectal cancer risk as well as a dose-dependent protective effect of modest levels of dietary folate supplementation (4-10X) above the basal dietary requirement on the development and progression of colorectal neoplasms (*11-15*). Animal studies have also shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels (*12, 16, 17*) and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa (*13, 14*) promote, rather than suppress, colorectal carcinogenesis. An accumulating body of evidence suggests that folate status may also play a modulatory role in the development of several other malignancies (e.g. lung, pancreas, stomach, cervix, esophagus, brain and leukemia) (*8-10*). The precise nature and magnitude of the relationship between folate status and the risk of these malignancies, however, are less clearly defined compared with colorectal cancer.

The relationship between folate status and breast cancer risk has just begun to be reported in the epidemiological literature. Among dietary factors implicated in the development of breast cancer, the inverse relationship between the consumption of vegetables and fruits (the major source of dietary folate) and breast cancer risk (*18*) and the positive correlation between the intake of alcohol (folate antagonist) and breast cancer risk (*19, 20*) have been most consistent (*1*). Among 13 published case-control studies that investigated the relationship between dietary folate intake and breast cancer risk, 10 showed either a significant or equivocal inverse relationship that was not statistically significant, that became nonsignificant after adjustment, or that could not be distinguished from other factors in their relation to risk (*21-30*), whereas 3 showed an unequivocal null association (*31-33*). In some studies, the observed inverse association was further modified by the intake of alcohol and other folate cofactors (e.g. methionine, vitamins $B_6$ and $B_{12}$) (*24, 26, 27*). Two large prospective studies (the Nurses' Health Study and the Iowa Women's study) have shown a weak inverse association between the total or dietary intake of folate and breast cancer risk (*34, 35*). These prospective studies, however, have indicated that low intakes of folate increase, whereas high intakes of folate decrease, breast

cancer risk among women who regularly consume alcohol (*34, 35*), supporting folate-alcohol interactions in breast carcinogenesis observed in case-control studies (*24, 26, 27*). However, two other large prospective studies (the American Cancer Society Cancer Prevention Study II Nutrition Cohort and the Nurses' Health Study II) found no association between the risk of breast cancer and dietary or total folate (*36, 37*) and no evidence of an interaction between folate and alcohol in modifying the risk of breast cancer (*37*). With respect to actual blood levels of folate and the risk of breast cancer, one nested case-control study, using stored serum samples, found no association between serum folate and breast cancer risk (*38*), whereas another nested case-control study (the Nurses' Health Study) demonstrated a weak inverse association between plasma folate levels and breast cancer risk, which was significantly modified by alcohol consumption (*39*). Overall, the portfolio of epidemiological evidence supporting the relationship between folate status and breast cancer risk is tenuous at best and does not provide unequivocal support for the purported inverse association, although a clearer picture emerges when studies examining the joint effect of folate and alcohol are considered.

Two animal studies published to date have produced conflicting results concerning the effect of folate on mammary tumorigenesis. In mice with confirmed spontaneous mammary cancers, daily intravenous injections of fermentation *Lactobacillus casei* factor (pteroyltriglutamate) significantly regressed mammary tumors and decreased new mammary tumor formation and lung metastases (*40*). Another study employing the *N*-methyl-*N*-nitrosourea (MNU) rat model showed that a folate-deficient diet provided during the initiation phase of mammary tumorigenesis significantly reduced tumor multiplicity and increased tumor latency compared with a control and folate-supplemented diet (*41*). The incidence of mammary tumors, however, was not significantly different among these groups (*41*). Several inherent limitations associated with these animal studies, however, preclude a definitive conclusion concerning the effect of folate on mammary tumorigenesis.

Because only few modifiable risk factors for breast cancer exist, recent epidemiological observations which suggest that folate deficiency increases, whereas supplementation reduces, breast cancer risk merit further consideration. Folate is an ideal agent for potential chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects (*42*), and possesses biologically plausible mechanisms for cancer prevention (*8-10*). However, the results from published epidemiological and animal studies have been neither consistent nor convincing. Furthermore, a growing body of evidence suggests that folate possesses the dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention (*11-17*). Folate deficiency has an inhibitory, whereas folate supplementation has a promoting, effect on progression of established neoplasms (*11-17*). By contrast, folate deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress the development of tumors in normal tissues (*11-17*). Therefore, the potential effect of folate chemoprevention needs to be clearly elucidated in appropriate animal models before folate supplementation can be considered in humans. Given these considerations, we proposed to study the effects of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the well-established MNU rat model of breast cancer in three animal experiments and to investigate potential molecular mechanisms by which dietary folate modulates mammary tumorigenesis. Notwithstanding the limitations associated with animal models, the MNU rat model is widely used to determine the effects of dietary factors on mammary tumorigenesis for the following reasons: (a) histological similarities of adenocarcinomas to human breast cancer; (b) local

invasiveness and metastatic potential; (c) a clear operational distinction between the initiation and promotion stages; and (d) hormonally dependent mammary tumorigenesis (*43-47*).

## BODY

**Task 1 (Specific Aim I):** To determine whether sustained folate deficiency of a moderate degree enhances, and whether a modest degree of folate supplementation above the basal requirement suppresses, the development of mammary tumors in the MNU rat model of mammary carcinogenesis (*initiation + promotion combined*)

We investigated the effect of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the MNU rat model. Weaning, female Sprague-Dawley rats were fed diets containing either 0 mg (deficient; n=22), 2 mg (basal dietary requirement, control; n=20) or 8 mg (supplemented; n=20) folate /kg diet for 30 weeks. At 50 days of age, rats received an intraperitoneal injection of MNU (50 mg/kg body weight). Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week. All rats were palpated for mammary tumors once a week beginning 4 weeks after MNU administration. The number, size and location of each tumor were recorded in a manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All the rats were monitored daily for clinical evidence of illness or morbidity and those approaching a moribund state were promptly euthanized. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15-20 mm in diameter, tumors that impaired normal movement of the animals, and ulcerating tumors were immediately euthanized during the study. Blood was collected from the tail of each rat within a week of MNU injection and from the heart at necropsy for the serum folate assay. Given the latency period of 3-6 months associated with a single intraperitoneal MNU injection and the average duration for the systemic and tissue folate levels to stabilize, the rats were sacrificed by carbon dioxide inhalation followed by cervical dislocation at 23 weeks after MNU injection (27 weeks after dietary intervention or 30 weeks of age). The liver from each rat was harvested for hepatic folate concentration determination. All macroscopic mammary tumors were counted, excised and weighed, and diameters of each tumor were measured using a digital caliper for final tumor volume computation in a blinded fashion. One-half of each macroscopic tumor was processed for DNA extraction. The other half of the tumor was processed in a standard manner for histological analysis according to Russo et al. (*46*) by three experienced pathologists blinded to the study group independently. In the case of a discrepancy, two similar interpretations were utilized for the final analysis. Normal mammary tissue was processed for DNA extraction and mammary tissue folate determination. Between-group comparisons of continuous variables were assessed using the Kruskal-Wallis and Mann-Whitney non-parametric tests. For categorical response variables, differences among the groups were assessed by Pearson chi-square test. The Kaplan-Meier survival analysis and the Log Rank test were used to compare the rates of tumor appearance among the three groups. All significance tests were two sided and were considered statistically significant if the observed significance level was $<0.05$. Results are expressed as mean ± SEM. Statistical analyses were performed using SPSS (version 10).

Serum folate concentrations accurately reflected dietary folate levels at the time of MNU administration and at necropsy ($P<0.001$). The mean folate concentrations of the normal mammary gland of the folate-deficient group were significantly lower than those of the control and folate-supplemented groups ($P<0.001$), whereas no significant difference between the

control and folate-supplemented groups was observed. The final incidence of mammary tumors in the folate-deficient group was significantly lower than that of the control and folate-supplemented groups (55% versus 90% and 75%, respectively, P=0.04). Kaplan-Meier analyses also demonstrated similar cumulative tumor incidence (or rates of tumor appearance) trends (P=0.06). By contrast, dietary folate supplementation did not significantly modulate both the final and cumulative incidences of mammary tumors compared with the control group. Dietary folate status had no significant effect on mean volume, weight, latency or multiplicity of mammary tumors. These data suggest that dietary folate deficiency of a moderate degree suppresses mammary tumorigenesis in this model. In contrast, dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammary tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer.

One abstract arising from Task 1 entitled "Dietary folate deficiency suppresses mammary tumorigenesis in a chemical carcinogen rat model of breast cancer" was presented in Poster Session: Chemoprevention, P13-8 at the Era of Hope meeting in Orlando, Florida on September 26, 2002 (**Appendix 1**). One manuscript arising from Task 1 entitled "Dietary folate deficiency suppresses N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats" was published in Carcinogenesis (*48*) (**Appendix 2**).

**Task 2 (Specific Aim II):** To determine whether folate deficiency enhances, and whether folate supplementation suppresses, the *initiation* of mammary carcinogenesis

In this animal experiment, we investigated the effect of dietary folate deficiency and supplementation on the *initiation* phase of mammary tumorigenesis in the MNU rat model. Based on the data obtained from Task 1, it is important to determine whether the inhibitory effective of dietary folate deficiency on mammary tumorigenesis in this model is during the initiation and/or promotion/progression phases of mammary tumorigenesis. In this experiment, similar to the animal experiment in Task 1, weaning, female Sprague-Dawley rats were fed diets containing either 0 mg (deficient; n=21), 2 mg (basal dietary requirement, control; n=20) or 8 mg (supplemented; n=20) folate /kg diet. At 50 days of age, rats received an intraperitoneal injection of MNU (50 mg/kg body weight). The initial diets were terminated one week following MNU-injection and all the rats were placed on the control (2 mg folate/kg diet) diet until the time of sacrifice (30 weeks of age). Animals were maintained and sacrificed, samples were harvested and prepared, and all assays and analyses were performed in the same manner as described in Task 1 (*48*).

Serum folate concentrations accurately reflected dietary folate levels at the time of MNU administration (P<0.001). At necropsy, serum and mammary gland folate concentrations of the three groups were not significantly different because the animals were placed on the control diet for 23 weeks after the MNU administration at 50 days of age. Dietary folate status during the initiation phase of MNU-induced mammary tumorigenesis had no significant effect on final and

cumulative incidences, latency, volume, weight or multiplicity of mammary tumors. These data indicate that dietary folate deficiency and supplementation do not significantly modulate the initiation phase of MNU-induced mammary tumorigenesis. One explanation for this observation is that, although the dose and route of MNU administration employed in this study may be appropriate in studies examining the effect of other potential chemopreventive agents in this model, the effect may be too overwhelmingly carcinogenic for folate to modulate. Regardless of the levels of dietary folate, MNU induced and established neoplastic foci in mammary tissues. The lack of effect of dietary folate on initiation of MNU-induced mammary tumorigenesis is, however, consistent with prior epidemiologic observations that have suggested that folate status alone may not be sufficient to modulate the development of breast cancer but only in conjunction with alcohol, with other folate co-factors, or with genetic predispositions (e.g. MTHFR C677T polymorphism). This is also in keeping with the similar observations pertaining to the role of folate in colorectal and uterine cervical carcinogenesis. These data suggest that the inhibitory effect of dietary folate deficiency on mammary tumorigenesis observed in Task 1 is primarily on promotion and progression, not on initiation, of MNU-induced neoplastic foci.

A manuscript combining the data from Task 2 and Task 3 has been prepared and will be submitted to Cancer Research (**Appendix 3**) as discussed in the next section.

**Task 3 (Specific Aim III):** To determine whether folate deficiency enhances, and whether folate supplementation suppresses, the *promotion* of mammary carcinogenesis

In this animal experiment, we investigated the effect of dietary folate deficiency and supplementation on the *promotion/progression* phase of mammary tumorigenesis in the MNU rat model. Based on the data obtained from Task 1, it is important to determine whether the suppressive effective of dietary folate deficiency on mammary tumorigenesis in this model is during the initiation and/or promotion/progression phases of mammary tumorigenesis. In this experiment, similar to the animal experiments in Task 1 and 2, weaning, female Sprague-Dawley rats (n=93) were placed on the control diet (2 mg folate/kg diet). At 50 days of age, rats received an intraperitoneal injection of MNU (50 mg/kg body weight). One week following MNU administration, rats were randomized to receive diets containing either 0 mg (deficient; n=33), 2 mg (basal dietary requirement, control; n=30) or 8 mg (supplemented; n=30) folate /kg diet until the time of sacrifice (30 weeks of age). Animals were maintained and sacrificed, samples were harvested and prepared, and all assays and analyses were performed in the same manner as described in Task 1 and 2 (*48*).

Serum folate concentrations of the three groups were not significantly different at the time of MNU-administration after being on the control diet for 50 days. However, at necropsy (30 weeks of age), serum folate concentrations of the three groups accurately reflected dietary folate levels after being placed on three diets containing different amounts of folate for 23 weeks after MNU-administration ($P<0.001$). The mean folate concentrations of the normal mammary gland of the folate-deficient group were significantly lower than those of the control and folate-supplemented groups ($P<0.001$), whereas no significant difference between the control and folate-supplemented groups was observed. The folate-deficient diet provided during the promotion phase of MNU-induced mammary tumorigenesis significantly reduced the final

incidence (52% versus 80% and 77%, respectively, P=0.027), cumulative incidence (P=0.03) and volume (P=0.01) of adenocarcinomas compared with the control and folate-supplemented diets, whereas no significant effects were observed for latency (P=0.53), weight (P=0.069) or multiplicity (P=0.18). Folate supplementation provided during the promotion phase had no significant effect on incidence and any of the parameters of adenocarcinomas compared with the control diet. Dietary folate status during the promotion phase had no significant effect on incidence and any of the parameters of adenomas. After neoplastic foci are established with MNU administration, our data from the promotion study clearly indicate that dietary folate deficiency of a moderate degree significantly suppressed the progression of or caused regression of established mammary neoplastic foci to adenocarcinomas. In contrast, dietary folate supplementation does not significantly modulate the progression of established mammary tumorigenesis.

Taken together, the data from Task 1, 2 and 3 collectively suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats and this effect is predominantly on the promotion/progression phase of MNU-induced mammary tumorgenesis. These observations suggest that the conventional dose and route of MNU injection employed in these studies have created an overwhelmingly carcinogenic milieu for folate status to modulate initiation of mammary tumorigenesis. Regardless of the levels of dietary folate, MNU induced and established neoplastic foci in mammary tissues. In this setting, folate deficiency suppressed the progression of and/or caused regression of established mammary neoplastic foci. This explanation is consistent with the prior observations made in Min and *Apc+/-Msh2-/-* mice with respect to intestinal tumorigenesis (*13, 14*). Also, this explanation is consistent with the known biochemical function of folate. As an essential co-factor for the de novo biosynthesis of purines and thymidylate, folate plays an important role in DNA synthesis and replication. Folate deficiency in tissues with rapidly replicating cells results in ineffective DNA synthesis. In neoplastic cells where DNA replication and cell divisions are occurring at an accelerated rate, interruption of folate metabolism causes ineffective DNA synthesis, resulting in inhibition of tumor growth. This has been the basis for cancer chemotherapy using antifolate agents. Therefore, the inhibitory effect of folate deficiency on MNU-induced mammary tumorigenesis in this rat model is primarily on promotion/progression of established mammary neoplastic foci. These animal studies in conjunction with prior observations made in animal models of colorectal cancer (*13, 14*) suggest that folate deficiency has an inhibitory effect on progression of established neoplasms. In contrast, folate supplementation may promote the progression of established intestinal neoplastic foci (*13, 14*), whereas the same degree of folate supplementation does not appear to promote the progression of established MNU-induced mammary neoplastic foci in our studies.

A manuscript incorporating the data from Task 2 and 3 has been prepared and will be submitted to Cancer Research (**Appendix 3**). A review article entitled "Role of folate in breast cancer development and progression: A critical review of epidemiologic and animal evidence" (authors: Kotsopoulos J, Kim YI) is being prepared to be submitted to Cancer Epidemiology Biomarkes Prevention, Journal of Nutrition or Cancer Causes Control by the end of November 2004.

**Task 4 (Specific Aim IV):** To determine molecular mechanisms by which folate status modulates mammary tumorigenesis in this model

Folate, in the form of 5-methyltetrahydrofolate, is involved in remethylation of homocysteine to methionine, which is a precursor of S-adenosylmethionine, the primary methyl group donor for most biological methylations including that of DNA (*8, 49, 50*). In this role, folate may modulate DNA methylation, which is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromosomal modifications and chromatin remodeling, and in the development of mutations (*51*). Neoplastic cells simultaneously harbor widespread genomic hypomethylation and more specific regional areas of hypermethylation (*51*). Genomic hypomethylation is an early, and consistent, event in carcinogenesis and is associated with genomic instability and increased mutations (*51*). Protooncogene-specific hypomethylation results in increased gene expression (*51*). In contrast, site-specific hypermethylation at promoter CpG islands of tumor suppressor and mismatch repair genes is an important mechanism in gene silencing in carcinogenesis (*51*). Given the role of folate in DNA methylation, which is generally considered an important epigenetic mechanism of cancer development, we investigated whether dietary folate modulates genomic DNA methylation in MNU-induced mammary tumorigenesis.

DNA from normal mammary tissues and mammary tumors was extracted by a standard technique using a lysis buffer containing proteinase K followed by phenol, chloroform, and isoamyl alcohol organic extraction (*52*). The size of DNA estimated by agarose-gel electrophoresis was >20 kb in all instances. No RNA contamination was detected on agarose-gel electrophoresis. The final preparations had a ratio of $A_{260}$ to $A_{280}$ between 1.8 and 2.0. The concentration of each DNA sample was determined as the mean of 3 independent spectrophotometric readings. The methylation status of cytosine-guanine (CpG) sites in genomic DNA from normal mammary tissues and mammary tumors was determined by the in vitro methyl acceptance capacity of DNA using [$^3$H-methyl]S-adenosylmethionine (SAM) as a methyl donor and a prokaryotic CpG DNA methyltransferase, Sss1, as previously described (*12, 14, 53, 54*). The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous [$^3$H-methyl] incorporation. Briefly, mammary tumor and non-neoplastic mammary gland DNA (500 ng) was incubated with 2.0 $\mu$Ci of [$^3$H-methyl]SAM (New England Nuclear, Boston, MA), 3 units of Sss1 methylase (New England Biolabs, Beverly, MA), and 1X Sss1 methylation buffer [120mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM EDTA, 1 mM dithiothreitol] in a total volume of 30 $\mu$L for 1 hour at 30°C. Sss1 was inactivated by incubating at 65°C for 10 minutes. The in vitro methylated DNA was isolated from a 15 $\mu$l aliquot of the reaction mixture by filtration on a Whatman DE-81 ion-exchange filter (Fisher Scientific, Springfield, NJ). The DNA was washed three times with 0.5 M sodium phosphate buffer (pH 7.0), air-dried and the radioactivity of the DNA retained in the filters was measured by scintillation counting using a nonaqueous scintillation fluor. The amount of radiolabel bound to a filter from an incubation mixture without DNA (control) was used as background and was subtracted from the values obtained with mixtures containing DNA. The background value was always <1% of the uptake observed with DNA samples. All analyses were performed in duplicate. Differences in genomic DNA methylation between normal mammary gland and tumor in each diet group was assessed by the Wilcoxon signed ranks test.

The degree of $^{3}$H-methyl incorporation into DNA of the mammary adenocarcinoma and into DNA from the pair-matched non-neoplastic mammary tissue was not significantly different among the three dietary groups in all three studies (Tasks 1, 2, and 3). In all three studies (Tasks 1, 2, and 3), however, the degree of $^{3}$H-methyl incorporation into DNA of the mammary adenocarcinomas, which is inversely related to the extent of genomic DNA methylation, was 4- to 5-fold higher than that of non-neoplastic mammary tissue within each dietary group, indicating a significantly lower degree of genomic DNA methylation in the adenocarcinomas compared with the normal mammary tissue ($P<0.04$).

Although promoter CpG islands hypermethylation of several genes including *BRCA1, ER, p16, E-Cadherin, TMS1, RASSF1*, leading to inactivation of these genes have been observed in human breast cancer (*55-60*), very few studies have reported genomic hypomethylation in human breast cancer (*61, 62*). To our knowledge, our study is the first to demonstrate that genomic DNA hypomethylation is an epigenetic phenomenon associated with MNU-induced mammary tumorigenesis in rats. The extent of genomic DNA methylation in mammary adenocarcinomas and in nonneoplastic mammary tissues was not significantly modulated by folate status. This observation suggests that altered genomic DNA methylation was not a likely mechanism by which folate deficiency suppressed mammary tumorigenesis in our study. Because both site-specific hypo- and hypermethylation play a role in carcinogenesis (*63, 64*) and because folate may modulate DNA methylation in a site-specific manner (*54*), however, the possibility that folate status may affect site-specific methylation of critical genes implicated in mammary tumorigenesis cannot be ruled out in our studies. Therefore, it would be of great interest to study site-specific methylation of protooncogenes and tumor suppressor genes implicated in mammary tumorigenesis (*2-4*) and how this is modulated by folate status. In this regard, we are working on site-specific DNA methylation of the ER gene and depending on the results of this analysis and we will proceed to determine protein expression of ER. Furthermore, we are working on RNA and protein expression and activity of both de novo and maintenance DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b) and demethylase at present.

The data concerning genomic mammary DNA methylation in MNU-mammary tumorigenesis in rats from Task 1 were incorporated into the published manuscript in Carcinogenesis (*48*) (**Appendix 2**). The data pertaining to DNA methyation from Tasks 2 and 3 have been incorporated into the manuscript to be submitted to Cancer Research (**Appendix 3**).

Folate is an essential co-factor for the de novo biosynthesis of puriens and thymidylate (*8, 65*). Therefore, folate plays an important role in DNA synthesis, stability and integrity, and repair, aberrations of which have been implicated in carcinogenesis (*8, 65*). A growing body of evidence from in vitro, animal, an dhuman studies indicates that folate deficiency is associate with DNA strand breaks, impaired DNA repair, and increased mutations, and that folate supplementation can correct some of these defects induced by folate deficiency (*8, 65*). Given these considerations, we investigated the effect of dietary folate deficiency and supplementation on mutations of the Ha-ras protooncogene. Between 80 – 90% of MNU-induced mammary tumors contain a single point mutation (G → A) in the 12$^{th}$ codon of the Ha-ras protooncogene (*66-68*). This Ha-ras mutation was determined by PCR-RFLP assay in histologically confirmed mammary adenocarcinomas from the three diet groups in all three Tasks as described previously by Dr. Michael Archer's group (collaborator) (*69*). Neoplastic foci were microdissected from

unstained slides in order to minimize contamination from normal tissues as described previously by us (*14, 70*). In all Tasks, the Ha-ras mutation rates ranged from 80 – 90% in the three dietary groups and the differences among the three groups were not statistically significant. No significant correlations were observed between the mutation rate and folate status (dietary intake, serum and mammary gland folate concentrations). This observation suggests that dietary folate deficiency and supplementation did not significantly modulate the development of the Ha-ras mutations in this model. We also determined the effects of dietary folate on microsatellite instability, the hallmark of DNA mismatch repair defect, in DNA from microdissected neoplastic foci and adjacent non-neoplastic tissue. Microsatellite instability was detected by comparison of electrophoretic mobility of amplified normal and tumor DNA using priemrs from loci on rat chromosomes 1 (D1MGH 22), 2 (D2MIT 12), 6 (IGHE), 10 (PPY), and X (AR) as previously described by us (*14, 71*). We analyzed 10 adenocarcinomas and pair-matched adjacent non-neoplastic mammary tissues from each of the three dietary groups in all three Tasks. No miscrosatellite instability was detected in any of the tumor samples or non-neoplastic mammary tissues we analyzed, suggesting that widespread microsatellite instability is likely not a major factor leading to the development of mammary tumors in this model. These data also suggest that superimposed folate deficiency and supplementation had no significant observable effect on microsatellite instability, suggesting that modulation of microsatellite instability is not likely a mechanism by which dietary folate modulates mammary tumorigenesis in this model.

In summary, the most likely molecular or biochemical mechanism by which dietary folate deficiency suppressed MNU-induced mammary tumorigenesis in this model was therefore the impaired de novo biosynthesis of purines and thymidylate in MNU-induced neoplastic foci, which resulted in ineffective DNA synthesis and replication leading to inhibition of the progression of neoplastic foci similar to cancer chemotherapy using antifolates such as methotrexate and 5-fluorouracil.

## KEY RESEARCH ACCOMPLISHMENTS

1. Our data from Task 1, 2 and 3 collectively indicate that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats, and this effect appears to be primarily via inhibition of the promotion/progression of established mammary neoplastic foci. Our data also indicate that dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis in this model. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer in humans. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammary tumorigenesis needs to be clarified in subsequent animal and human studies for safe and effective prevention of breast cancer.
2. Our study is the first to demonstrate that genomic DNA hypomethylation is an epigenetic phenomenon associated with MNU-induced mammary tumorigenesis. However, the extent of genomic DNA methylation in mammary tumors was not significantly modulated by folate status. This observation suggests that altered genomic DNA methylation was not a likely mechanism by which folate deficiency suppressed mammary tumorigenesis in this study.

## REPORTABLE OUTCOMES

1. Kotsopoulos J, Sohn K-J, Martin R, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Dietary folate deficiency suppresses mammary tumorigenesis in a chemical carcinogen rat model of breast cancer. (Poster presentation at Chemoprevention, P13-8 at Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orlando, Florida, September 25 – 28, 2002; **Appendix 1**)
2. Kotsopoulos J, Sohn K-J, Martin R, Choi M, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Dietary folate deficiency suppresses N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats. Carcinogenesis 2003; 24: 937 – 944 (**Appendix 2**)
3. Kotsopoulos J, Medline A, Renlund R, Sohn KJ, Martin R, Hwang SW, Lu S, Archer MC, **Kim YI**. Effects of dietary folate on the development and progression of mammary tumors in rats. Cancer Research (to be submitted; **Appendix 3**)

## CONCLUSIONS

Our data from Task 1, 2 and 3 collectively indicate that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats and that this effect is predominantly on the promotion/progression phase of MNU-induced mammary tumorigenesis. Our data also indicate that dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis in this model. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer in humans. However, epidemiologic evidence available thus far has not been consistent nor has it provided unequivocal support for the purported inverse relationship between folate status and breast cancer risk. Some epidemiologic studies have suggested that folate status alone may not be sufficient in modifying breast cancer risk. However, with alcohol consumption, a well-established risk factor for breast cancer development, folate deficiency potentiates, whereas folate supplementation reduces, the risk of breast cancer. Furthermore, some studies have suggested that folate status may modify breast cancer risk in conjunction with other dietary factors involved in one-carbon metabolism such as methionine, vitamins $B_6$ and $B_{12}$. Also, there is evidence that the direction and magnitude of the breast cancer risk modification associated with folate status may depend on the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene that encodes an enzyme critical for intracellular folate homeostasis.

The strengths of the present study include: (i) the use of the amino acid-defined diet that is widely accepted as the standard means of inducing folate deficiency or providing supplemental dietary folate in rodents; (ii) the use of dietary levels of folate that have been shown to modulate development of other cancers in this strain of rats; (iii) measurements of systemic and mammary gland folate concentrations; and (iv) rigorous histological confirmation of all mammary tumors to ensure an accurate determination of the rate of appearance and other tumor-specific parameters of adenomas and adenocarcinomas. However, several limitations associated with the present study need to be acknowledged. First, although the dose and route of MNU administration employed in the present study may be appropriate in studies examining the effect of other potential chemopreventive agents in this model, the effect may be too overwhelmingly carcinogenic for folate to modulate. Regardless of the levels of dietary folate, MNU likely induced and established neoplastic foci in mammary tissues. Therefore, only the effect of dietary folate on promotion and progression, but not on initiation, of MNU-induced neoplastic foci may be determined in this model. Second, the fat content of the diets used in the present study was higher than the AIN rodent diets that are more commonly used in experimental mammary tumor studies (10% versus 7% by weight). Animal studies have generally suggested that high fat diets enhance mammary tumorigenesis in rodents. Therefore, it is possible that the tumor-promoting effect associated with the higher fat content in our diets might have confounded the modulating effect of dietary folate intervention. Third, the mean mammary gland folate concentration associated with folate supplementation was not significantly higher than that of the control diet. Therefore, higher levels of folate supplementation above 4x the basal dietary requirement may be necessary to significantly increase mammary gland folate concentrations in order to observe any modulatory effect of folate supplementation.

Notwithstanding the limitations associated with this model, our data, in conjunction with the portfolio of epidemiologic evidence, suggest that the role of folate in mammaray tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer. In particular, interactions between folate and alcohol and between folate and other folate cofactors in modifying breast cancer risk merit further consideration. Also, the effect of genetic polymorphisms in the folate and alcohol metabolic pathways on breast cancer risk and related gene-folate interactions in further modulating this effect need to be clearly elucidated. These studies will lead to more rational and logical strategies using folic acid to prevent breast cancer. For instance, individuals with the MTHFR 677TT genotype with inadequate folate intake or with significant alcohol consumption have been shown to have an increased risk of breast cancer. These individuals may therefore benefit from targeted folic acid chemoprevention. Given the emerging body of evidence that suggests the dual modulatory role of folate on carcinogenesis depending on the timing and dose of folate intervention and the lack of convincing evidence for the protective effect of folate supplementatin on breast cancer risk, however, the use of folic acid supplementation to prevent breast cancer should not be recommeded at present. Future studies employing lower doses of MNU, lower fat content and higher levels of folate supplementation may be necessary to clearly elucidate the effect of folate on mammary tumorigenesis in this model. The effect of folate on mammary tumorigenesis observed in the present study needs to be confirmed in other animal models. Our data concerning genomic DNA methylation also indicate that genomic mammary DNA hypomethylation is an epigenetic mechanism by which mammary tumors develop in the MNU rat model. Studies are underway to investigate site and gene-specific DNA methylation changes in this model. Our data also suggest that changes in genomic mammary DNA methylation are not a likely mechanism by which folate modulates mammary tumorigenesis in this model.

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## APPENDICES

1. Kotsopoulos J, Sohn K-J, Martin R, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Dietary folate deficiency suppresses mammary tumorigenesis in a chemical carcinogen rat model of breast cancer. (poster presentation at Chemoprevention, P13-8 at Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orlando, Florida, September 25 – 28, 2002)
2. Kotsopoulos J, Sohn K-J, Martin R, Choi M, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Dietary folate suppresses N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats. Carcinogenesis 2003; 24; 937 – 944.
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# DIETARY FOLATE DEFICIENCY SUPPRESSES MAMMARY TUMORIGENESIS IN A CHEMICAL CARCINOGEN RAT MODEL OF BREAST CANCER

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Epidemiological studies have suggested that dietary folate intake is inversely related to the risk of breast cancer. This study investigated the effect of dietary folate on the development and progression of N-methyl-N-nitrosourea (MNU)-induced mammary tumorigenesis in rats. Weanling, female Sprague-Dawley rats were randomized to receive an amino acid-defined diet containing either 0 mg (moderately folate deficient; n=22), 2 mg (basal dietary requirement [control]; n=20) or 8 mg (supplemented; n=20) folate/kg diet. At 50 days of age, all the rats received an intraperitoneal injection of MNU (50 mg/kg body weight) and the initial dietary interveniton was continued for additional 23 weeks. At necropsy, all macroscopic mammary tumors were identified and histologically confirmed for adenocarcinoma or its precursor, adenoma. Serum folate concentrations accurately reflected dietary folate levels at the time of MNU administration and at necropsy ($P<0.001$). The mean folate concentrations of the normal mammary gland of the folate-deficient group were significantly lower than those of the control and folate-supplemented groups ($P<0.001$), whereas no significant difference between the control and folate-supplemented groups was observed. The final incidence of mammary tumors in the folate-deficient group was significantly lower than that of the control and folate-supplemented groups (55% versus 90% and 75%, respectively, $P=0.04$). Kaplan-Meier analyses also demonstrated similar cumulative tumor incidence trends ($P=0.06$). By contrast, dietary folate supplementation did not significantly modulate both the final and cmulative incidences of mammary tumors compared with the control group. Dietary folate status had no significant effect on mean volume, weight, latency or multiplicity of mammary tumors. These data suggest that dietary folate deficiency of a moderate degree suppresses mammary tumorigenesis in this model. By contrast, dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis. The role of folate in mammaray tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer.

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# Dietary folate deficiency suppresses *N*-methyl-*N*-nitrosourea-induced mammary tumorigenesis in rats

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**Epidemiologic studies have suggested that dietary folate intake is inversely related to breast cancer risk. However, epidemiologic evidence has not been consistent nor has it provided unequivocal support for this purported inverse relationship. This study investigated the effect of dietary folate on *N*-methyl-*N*-nitrosourea (MNU)-induced mammary tumorigenesis in rats. Weanling, female Sprague–Dawley rats were fed diets containing either 0 (deficient; $n = 22$), 2 (basal dietary requirement, control; $n = 20$) or 8 mg (supplemented; $n = 20$) folate/kg diet for 30 weeks. At 50 days of age, rats received an i.p. injection of MNU (50 mg/kg body wt). At necropsy, all macroscopic mammary tumors were identified and examined microscopically. The effect of dietary folate on genomic DNA methylation in mammary tumorigenesis was determined by the *in vitro* methyl acceptance assay. The incidence of mammary adenoma and adenocarcinoma in the folate-deficient group was lower than that of the control and folate-supplemented groups (55 versus 90 and 75%, respectively, $P = 0.043$). Kaplan–Meier analyses also demonstrated a similar trend in the rates of appearance of either adenoma or adenocarcinoma ($P = 0.06$). In contrast, folate supplementation did not significantly modulate mammary tumorigenesis compared with the control group. Although mammary tumors were significantly hypomethylated compared with non-neoplastic mammary tissues in each dietary group ($P < 0.03$), folate status did not significantly affect the extent of DNA methylation. The data suggest that dietary folate deficiency of a moderate degree suppresses, whereas folate supplementation at four times the basal dietary requirement does not significantly modulate, mammary tumorigenesis in this model. The role of folate in mammary tumorigenesis needs to be clarified for safe and effective prevention of breast cancer.**

## Introduction

Folate, a water-soluble B-vitamin and important co-factor in one-carbon metabolism, has recently been identified as an important nutritional factor that may modulate carcinogenesis (1–3). The role of folate in carcinogenesis has been best studied for colorectal cancer (1–3). The majority of over 25 published epidemiological studies indicate that dietary folate intake and blood folate levels are inversely associated with colorectal cancer risk (1–3). Although animal studies are generally supportive of a causal relationship between folate depletion and colorectal cancer risk, these studies have shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels (4–6) and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa (7,8) promote, rather than suppress, colorectal carcinogenesis. An accumulating body of evidence suggests that folate status may also play a modulatory role in the development of several other malignancies (e.g. lung, pancreas, stomach, cervix, esophagus, brain and leukemia) (1–3). The precise nature and magnitude of the relationship between folate status and the risk of these malignancies, however, are less clearly defined compared with colorectal cancer.

The relationship between folate status and breast cancer risk has just begun to be reported in the epidemiological literature. Among nine published case-control studies that investigated the relationship between dietary folate intake and breast cancer risk, seven showed either a significant or equivocal inverse relationship that was not statistically significant, that became non-significant after adjustment, or that could not be distinguished from other factors in their relation to risk (9–15), whereas two showed an unequivocal null association (16,17). In some studies, the observed inverse association was further modified by the intake of alcohol and other folate co-factors (e.g. methionine, vitamins $B_6$ and $B_{12}$) (12,14,15). One nested case-control study, using stored serum samples, found no association between serum folate and breast cancer risk (18). Two large prospective studies have shown a weak inverse association between the total or dietary intake of folate and breast cancer risk (19,20). These prospective studies, however, have indicated that low intakes of folate increase, whereas high intakes of folate decrease, breast cancer risk among women who regularly consume alcohol (19,20), supporting folate–alcohol interactions in breast carcinogenesis observed in case-control studies (12,14,15). Recently, molecular epidemiologic studies have shown that the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene may modulate breast cancer risk and that the direction and magnitude of the risk modification are influenced by folate status and alcohol consumption (21–23). MTHFR is a critical enzyme in folate metabolism that catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thereby playing an important role in DNA

**Abbreviations:** FPGS, folylpolyglutamate synthetase; MTHFR, methylenetetrahydrofolate reductase; MNU, *N*-methyl-*N*-nitrosourea.





Appendix 2

synthesis, maintenance of nucleotide pool balance and DNA methylation (1). The MTHFR C677T polymorphism causes thermolability and reduced MTHFR activity, leading to lower levels of 5-methyltetrahydrofolate, an accumulation of 5,10-methylenetetrahydrofolate, increased plasma homocysteine levels (a sensitive inverse indicator of folate status), changes in cellular composition of one-carbon folate derivatives, and DNA hypomethylation (1).

Two animal studies published to date have suggested that folate may modulate mammary tumorigenesis. In mice with confirmed spontaneous mammary cancer, daily i.v. injections of fermentation *Lactobacillus casei* factor (pteroyltriglutamate) significantly regressed pre-existing mammary tumors and decreased new mammary tumor formation and lung metastases (24). Another study employing the *N*-methyl-*N*-nitrosourea (MNU) rat model showed that a folate-deficient diet provided during the initiation phase of mammary tumorigenesis significantly reduced tumor multiplicity and increased tumor latency compared with a control and folate-supplemented diet (25). The incidence of mammary tumors, however, was not significantly different among these groups (25). However, several inherent limitations associated with these animal studies including the use of non-standard dietary means to modulate folate status, possible growth retardation of animals, the concomitant use of antibiotics that may independently affect folate levels, and the use of animals that are resistant to chemically induced mammary tumorigenesis preclude a definitive conclusion concerning the effect of folate on mammary tumorigenesis.

Because only few modifiable risk factors for breast cancer exist, recent epidemiological observations which suggest that folate deficiency increases, whereas supplementation reduces, breast cancer risk merit further consideration. Folate is an ideal agent for potential chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects (26) and possesses biologically plausible mechanisms for cancer prevention (1–3). However, the results from published epidemiological and animal studies have been neither consistent nor convincing. Furthermore, a growing body of evidence suggests that folate possesses the dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention (4–8,27,28). Folate deficiency has an inhibitory, whereas folate supplementation has a promoting, effect on progression of established neoplasms (4–8,27,28). In contrast, folate deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress the development of tumors in normal tissues (4–8,27,28). Therefore, the potential effect of folate chemoprevention needs to be clearly elucidated in appropriate animal models before folate supplementation can be considered in humans. Given these considerations, this study investigated the effects of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the well-established MNU rat model of breast cancer. Given the role of folate in DNA methylation, an important epigenetic determinant in carcinogenesis (29,30), we also investigated whether dietary folate modulates genomic DNA methylation in MNU-induced mammary tumorigenesis. Folate, in the form of 5-methyltetrahydrofolate, is involved in remethylation of homocysteine to methionine, which is a precursor of *S*-adenosylmethionine (SAM), the primary methyl group donor for most biological methylation reactions (1–3).

## Materials and methods

*Animals and dietary intervention*

This study was approved by the Animal Care Committee of the University of Toronto. Pathogen-free, weanling female Sprague–Dawley rats (~50 g; Charles River Laboratories, St Constant, Quebec, Canada) were randomly assigned to receive an amino acid-defined diet (Dyets, Bethlehem, PA) (31) containing either 0 ($n = 22$), 2 ($n = 20$) or 8 ($n = 20$) mg folic acid/kg diet from weaning at 3 weeks of age for 27 weeks through the MNU treatment (at 50 days of age). Rats were singly housed and maintained at 24 ± 2°C at 50% humidity with a 12 h light/dark cycle. These diets constitute a standard method of inducing folate deficiency or providing supplemental dietary folate in rodents (31) and have been utilized extensively in previous studies of folate and colorectal cancer (4,7,8,27). The diet containing 0 mg folic acid/kg produces progressive folate deficiency of a moderate degree without anemia, growth retardation or premature death through weeks 3–5, after which systemic folate indicators stabilize (27). Although this diet is completely devoid of folate, severe folate deficiency is not induced because of de novo synthesis of folate by intestinal bacteria, some of which is incorporated into the tissue folate of the host (32). This folate-deficient diet is identical to that associated with an increased risk of colorectal neoplasms in previous animal studies using a chemical colorectal carcinogen or genetically engineered murine models of colorectal cancer (4,7,8,27). Two milligram folic acid per kilogram diet is generally accepted as the basal dietary requirement for rodents (33). The diet containing 8 mg folic acid/kg represents folate supplementation four times the basal dietary requirement. This level of folate was chosen because the 8 mg/kg level has consistently provided a degree of chemoprevention against colorectal cancer in previous rodent studies (4,7,27). These diets contained 50 g cellulose/kg, 60% of the calories as carbohydrates, 23% as fat (or 10% by weight), and 17% as L-amino acids (31). The amount of methyl donors, methionine, choline and vitamin $B_{12}$, 8.2 g, 2.0 g and 50 μg/kg diet, respectively. The detailed composition of the diets has been published previously (8,31). Diets and water were provided *ad libitum*.

*MNU administration*

Notwithstanding the limitations associated with animal models, the MNU rat model is widely used to determine the effects of dietary factors on mammary tumorigenesis for the following reasons: (i) histological similarities of adenocarcinoma to human breast cancer; (ii) local invasiveness and metastatic potential; (iii) a clear operational distinction between the initiation and promotion stages; and (iv) hormonally dependent mammary tumorigenesis (34–38). At 50 days of age, all rats received one i.p. injection of MNU (50 mg/kg body wt; Sigma Chemical, St Louis, MO). A single injection of 50 mg MNU/kg has become the standard dosage due to its rapid induction and high incidence of mammary tumors combined with minimal toxicity and a short latency period of 3–6 months (34,35).

*Observation parameters*

Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week. All rats were palpated for mammary tumors once a week beginning 4 weeks after MNU administration. The number, size and location of each tumor were recorded in a manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All the rats were monitored daily for clinical evidence of illness or morbidity and those approaching a moribund state were promptly killed. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15–20 mm in diameter, tumors that impaired normal movement of the animals and ulcerating tumors were immediately killed during the study.

*Sample collection and analysis of mammary tumors*

Blood was withdrawn from the lateral tail vein of each rat within a week of MNU injection and from the heart at necropsy and centrifuged at 5000 r.p.m. for 10 min at 4°C. Serum was stored at −70°C in 0.5% ascorbic acid for serum folate assay. Given the latency period of 3–6 months associated with a single i.p. MNU injection and the average duration for the systemic and tissue folate levels to stabilize, the rats were killed by carbon dioxide inhalation followed by cervical dislocation at 23 weeks after MNU injection (27 weeks after dietary intervention or 30 weeks of age). The liver from each rat was harvested, snap-frozen and stored at −70°C for determination of hepatic folate concentration. All macroscopic mammary tumors were counted, excised and weighed, and the diameter of each tumor was measured using a digital caliper for final tumor volume computation in a blinded fashion. One-half of each macroscopic tumor was snap-frozen in liquid nitrogen and stored at −70°C for DNA extraction. The other half of the tumor was fixed in 10% neutral-buffered formalin, processed in a standard manner for hematoxylin–eosin (H&E) staining and histologically analyzed according to Russo *et al.* (37) by three experienced pathologists (R.R., C.M. and A.M.) blinded to the study group independently.

In the case of a discrepancy, two similar interpretations were utilized for the final analysis. Normal mammary tissue was also excised at necropsy from each rat, snap-frozen in liquid nitrogen and stored at −70°C for DNA extraction and mammary tissue folate determination.

*Determination of folate concentration*

Serum folate concentrations were determined by a standard microbiological microtiter plate assay using *L.casei* (39). Hepatic and normal mammary tissue folate concentrations were measured by the same microbiologic assay (39), utilizing a previously described method for the determination of tissue folates (40).

*DNA extraction*

DNA from normal mammary tissue and mammary tumors was extracted by standard technique using a lysis buffer containing proteinase K followed by phenol, chloroform and isoamyl alcohol organic extraction (41). The size of DNA estimated by agarose-gel electrophoresis was >20 kb in all instances. No RNA contamination was detected on agarose-gel electrophoresis. The final preparations had a ratio of $A_{260}$ to $A_{280}$ between 1.8 and 2.0. The concentration of each DNA sample was determined as the mean of three independent spectrophotometric readings.

*Genomic DNA methylation determination*

The methylation status of cytosine-guanine (CpG) sites in genomic DNA from normal mammary tissue and mammary tumors was determined by the *in vitro* methyl acceptance capacity of DNA using $^3$H-methyl-SAM as a methyl donor and a prokaryotic CpG DNA methyltransferase, Sss1, as described previously (4,8,42,43). The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous $^3$H-methyl incorporation. Briefly, mammary tumor and non-neoplastic mammary gland DNA (500 ng) was incubated with 2.0 μCi of $^3$H-methyl-SAM (New England Nuclear, Boston, MA), 3 U Sss1 methylase (New England Biolabs, Beverly, MA), and 1× Sss1 methylation buffer [120 mM NaCl, 10 mM Tris–HCl (pH 7.9), 10 mM EDTA, 1 mM dithiothreitol] in a total volume of 30 μl for 1 h at 30°C. The Sss1 was inactivated by incubating at 65°C for 10 min. The *in vitro* methylated DNA was isolated from a 15 μl aliquot of the reaction mixture by filtration on a Whatman DE-81 ion-exchange filter (Fisher Scientific, Springfield, NJ). The DNA was washed three times with 0.5 M sodium phosphate buffer (pH 7.0), air-dried and the radioactivity of the DNA retained in the filters was measured by scintillation counting using a non-aqueous scintillation fluor. The amount of radiolabel bound to a filter from an incubation mixture without DNA (control) was used as background and was subtracted from the values obtained with mixtures containing DNA. The background value was always <1% of the uptake observed with DNA samples. All analyses were performed in duplicate.

*Statistical analysis*

Between-group comparisons of continuous variables were assessed using the Kruskal–Wallis and Mann–Whitney non-parameteric tests. For categorical response variables, differences among the groups were assessed by Pearson $\chi^2$. Differences in genomic DNA methylation between normal mammary gland and tumor in each diet group was assessed by the Wilcoxon signed ranks test. The Kaplan–Meier survival analysis and the Log Rank test were used to compare the rates of tumor appearance among the three groups. All significance tests were two sided and were considered statistically significant if the observed significance level was <0.05. Results are expressed as mean ± SEM. Statistical analyses were performed using SPSS (version 10).

## Results

*Body weight and daily food consumption*

Growth curves were similar among the three dietary groups; at no time point did the mean body weights differ significantly among the three groups. This finding indicates that folate deficiency in the rats fed 0 mg folate/kg diet was moderate; otherwise, growth retardation or premature death would have occurred (44). The mean daily food consumption, which was determined on a pre-assigned day of each week, was also similar among the three groups.

*Serum, liver and normal mammary gland folate concentrations*

At the time of MNU injection (4 weeks after the start of dietary intervention) and at necropsy (27 weeks after the start of dietary intervention), the mean serum folate concentrations were significantly different among the three groups ($P < 0.001$; Table I). The mean serum folate concentrations of the three dietary groups at these two time points were comparable with those observed in rats and mice placed on the corresponding diets for 20–24 weeks in previous studies (4,7,27,45). These observations indicate that a sufficient degree of systemic folate deficiency and supplementation was achieved in the folate-deficient and supplemented rats, respectively, at the time of MNU injection and throughout the study period for the determination of the effect of folate status on MNU-induced mammary tumorigenesis. At necropsy, the hepatic folate concentrations of the three dietary groups were significantly different ($P < 0.001$; Table I), and these levels were comparable with those observed in rats placed on the corresponding diets for 24 weeks in previous studies (27,46). At necropsy, the mean mammary gland folate concentration of the folate-deficient group was significantly lower than the control and folate-supplemented groups ($P < 0.001$) while no significant difference was observed between the control and folate-supplemented groups (Table I). This observation suggests that mammary gland folate concentrations reached a plateau beyond the 2 mg folate/kg diet. This finding is probably due to the fact that folate accumulation in tissues is limited by the level of folylpolyglutamate synthetase (FPGS) activity in the setting of substrate excess (47,48).

*Effects of dietary folate on MNU-induced mammary tumorigenesis*

No rats died prematurely or were killed before necropsy in the three dietary groups for reasons other than the presence of large and/or ulcerating tumors as defined in the Materials and methods section. The prevalence of killed rats was similar among the three groups. Consistent with previous observations made in the MNU-Sprague–Dawley rat model of mammary tumorigenesis (34–38), >90% of macroscopic mammary tumors in the present study were identified histologically as either adenomas (15%) or adenocarcinomas (85%). There was an excellent agreement in histological diagnosis of either adenoma or adenocarcimona among the three study pathologists (kappa statistic = 0.95). The analyses pertaining to mammary tumors were performed for the combination of

**Table I.** Serum, hepatic and mammary gland folate concentrations*

| | At the time of MNU injection (4 weeks of dietary intervention) | | | At necropsy (27 weeks of dietary intervention) | | |
|---|---|---|---|---|---|---|
| Diet (mg folate/kg diet) | 0 | 2 | 8 | 0 | 2 | 8 |
| Serum folate (ng/ml) | 13.75 ± 1.04[a] | 68.82 ± 2.65[b] | 100.78 ± 2.82[c] | 10.53 ± 1.16[a] | 49.24 ± 3.16[b] | 77.35 ± 2.33[c] |
| Hepatic folate (μg/g tissue) | | | | 2.18 ± 0.32[a] | 6.42 ± 0.35[b] | 9.01 ± 0.37[c] |
| Mammary folate (ng/g tissue) | | | | 56.20 ± 7.54[a] | 178.13 ± 29.06[b] | 175.94 ± 24.32[b] |

*Results are expressed as mean ± SEM. Means in a row with different letters at each time point significantly differ at $P < 0.001$.

adenocarcinomas and adenomas and for adenocarcinomas alone. There were not a sufficient number of adenomas for independent analysis.

As shown in Figure 1A, there was a trend towards a significant difference in the rates of appearance of either adenocarcinomas or adenomas among the three dietary groups ($P = 0.07$). This was mainly due to the difference between the folate-deficient and control groups ($P = 0.02$). In contrast, there was no significant difference between the folate-deficient and supplemented groups ($P = 0.11$), and between the control and folate-supplemented groups ($P = 0.72$). We excluded one outlier from the folate-deficient group, which harbored a total of nine adenocarcinomas and adenomas, and this strengthened the overall comparison ($P = 0.06$). When the analysis was confined to adenocarcinomas alone, similar patterns were observed. There was a trend towards a significant difference in the rates of appearance of adenocarcinomas among the three groups ($P$-overall = 0.08; $P = 0.05$ between the folate-deficient and control groups; $P = 0.04$ between the folate-deficient and supplemented groups; $P = 0.83$ between the control and folate-supplemented groups; Figure 1B).

There was a trend towards a significant difference in the final incidence of histologically confirmed adenocarcinomas and adenomas at necropsy ($P = 0.057$; Table II). This was mainly due to the difference between the folate-deficient and control groups ($P = 0.02$); there was no significant difference between the folate-deficient and supplemented groups ($P = 0.19$) or between the control and folate-supplemented groups ($P = 0.20$). When the outlier was excluded from the folate-deficient group, the overall difference in the incidence of adenocarcinomas and adenomas became significant ($P = 0.043$) with a similar trend in between-group comparisons. As shown in Table II, there was no significant difference in mean tumor latency (mean time to appearance of first palpable tumor), multiplicity (mean number of tumors per tumor-bearing rat), volume or weight among the three groups, whether or not the outlier was excluded in the analyses. When the analyses were confined to adenocarcinomas alone, no

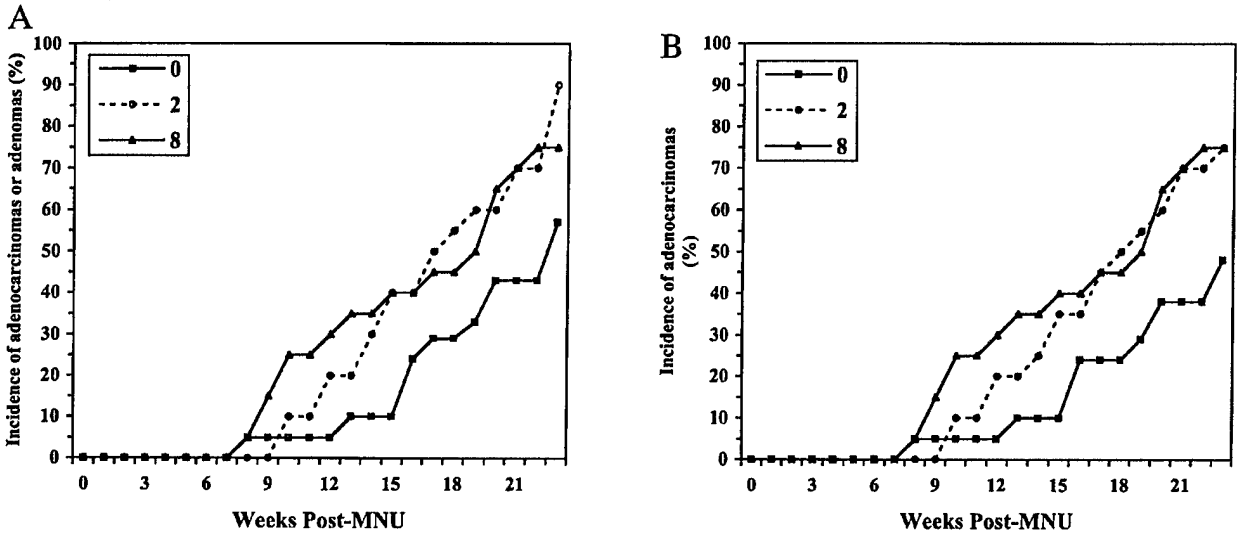


**Fig. 1.** (A) The rate of appearance of either mammary adenomas or adenocarcinomas among the three dietary groups ($P$-overall = 0.07; $P = 0.02$ between the 0 and 2 mg folic acid groups; $P = 0.11$ between the 0 and 8 mg folic acid groups; $P = 0.72$ between the 2 and 8 mg folic acid groups by the Kaplan–Meier survival analysis and Log Rank test). Excluding one outlier in the 0 mg folic acid group, which harbored a total of nine adenocarcinomas and adenomas, strengthened the overall comparison ($P = 0.06$) with similar patterns in between-groups comparisons ($P = 0.02$ between the 0 and 2 mg folic acid groups; $P = 0.09$ between the 0 and 8 mg folic acid groups; $P = 0.72$ between the 2 and 8 mg folic acid groups). (B) The rate of appearance of mammary adenocarcinomas among the three dietary groups ($P$-overall = 0.08; $P = 0.05$ between the 0 and 2 mg folic acid groups; $P = 0.04$ between the 0 and 8 mg folic acid groups; $P = 0.83$ between the 2 and 8 mg folic acid groups by the Kaplan–Meier survival analysis and Log Rank test).

**Table II.** Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary adenocarcinomas and adenomas*

| Diet (mg folate/kg diet) | 0 | 2 | 8 | $P$-value, ANOVA |
|---|---|---|---|---|
| Incidence (%) | 57[a] | 90[b] | 75[a,b] | 0.057 |
| Incidence (%)† | 55[a] | 90[b] | 75[a,b] | 0.043 |
| Mean latency (weeks post-MNU injection) | 17.83 ± 1.35 | 17.06 ± 1.11 | 15.00 ± 1.36 | 0.29 |
| Mean multiplicity | 3.67 ± 1.03 | 2.87 ± 0.53 | 2.20 ± 0.34 | 0.72 |
| Mean volume ($cm^3$) | 2.83 ± 0.80 | 2.88 ± 0.58 | 1.49 ± 0.39 | 0.28 |
| Mean weight (g) | 0.86 ± 0.23 | 1.03 ± 0.23 | 0.58 ± 0.14 | 0.45 |

*Results are expressed as mean ± SEM. Means in a row with different letters significantly differ at $P < 0.02$ by between-group comparisons.
†Excluding one outlier in the 0 mg folate group, which harbored a total of nine adenocarcinomas and adenomas, strengthened the overall comparison of the incidence of adenocarcinomas and adenomas among the three groups. In contrast, no significant difference in mean latency, multiplicity, volume and weight of adenocarcinomas and adenomas was observed among the three groups whether or not the outlier was included or excluded in the analyses.

**Table III.** Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary adenocarcinomas*

| Diet (mg folate/kg diet) | 0 | 2 | 8 | *P*-value, ANOVA |
|---|---|---|---|---|
| Incidence (%) | 48 | 75 | 75 | 0.10 |
| Mean latency (weeks post-MNU injection) | 17.40 ± 1.54 | 16.27 ± 1.10 | 15.00 ± 1.36 | 0.51 |
| Mean multiplicity | 3.71 ± 1.04 | 3.00 ± 0.59 | 1.90 ± 0.29 | 0.21 |
| Mean volme ($cm^3$) | 3.15 ± 0.99 | 3.10 ± 0.65 | 1.64 ± 0.42 | 0.38 |
| Mean weight (g) | 0.98 ± 0.28 | 1.12 ± 0.25 | 0.63 ± 0.15 | 0.42 |

*Results are expressed as mean ± SEM. No significant difference in mean latency, multiplicity, volume and weight of adenocarcinomas was observed among the three groups.

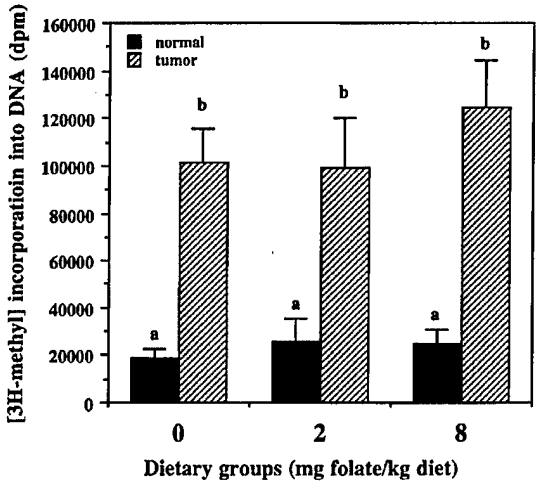


**Fig. 2.** Effects of dietary folate on genomic DNA methylation in mammary adenocarcinomas and non-neoplastic mammary tissues as determined by the *in vitro* methyl acceptance assay. The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous $^3$H-methyl incorporation into DNA. Different letters within each dietary group denote significant differences by the Wilcoxon signed ranks test at $P < 0.03$. Values are mean ± SEM.

significant difference in the final incidence and mean tumor latency, multiplicity, volume or weight among the three groups was observed (Table III).

*Genomic DNA methylation status*

As shown in Figure 2, the degree of $^3$H-methyl incorporation into DNA of the mammary adenocarcinoma and into DNA from the pair-matched non-neoplastic mammary tissue was not significantly different among the three dietary groups. However, the degree of $^3$H-methyl incorporation into DNA of the mammary adenocarcinomas, which is inversely related to the extent of genomic DNA methylation, was 4–5-fold higher than that of non-neoplastic mammary tissue within each dietary group ($P < 0.03$; Figure 2), indicating a significantly lower degree of genomic DNA methylation in adenocarcinomas compared with normal mammary tissue.

## Discussion

Our data suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats. In contrast, dietary folate supplementation at four times the basal dietary requirement does not appear to modulate mammary tumorigenesis in this model. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer (9–15,19,20). Epidemiologic evidence available thus far has not been consistent nor has it provided unequivocal support for the purported inverse relationship between folate status and breast cancer risk (9–20). However, none of the published epidemiologic studies has demonstrated a positive association between folate status and breast cancer risk. Some epidemiological studies have suggested that folate status alone may not be sufficient in modifying breast cancer risk. However, with alcohol consumption folate deficiency potentiates, whereas folate supplementation reduces, the risk of breast cancer (12,14,15,19,20). Furthermore, some sudies have suggested that folate status may modify breast cancer risk in conjunction with other dietary factors involved in one-carbon metabolism such as methionine, vitamins $B_6$ and $B_{12}$ (15,19). Also, there is evidence that the direction and magnitude of the breast cancer risk modification associated with folate status may depend on the MTHFR C677T polymorphism (21–23).

Our data differ from the promoting and protective effect of folate deficiency and supplementation, respectively, on intestinal tumorigenesis observed in the chemical carcinogen (dimethylhydrazine) and genetically engineered rodent models utilizing the same diets employed in the present study (4,7,27,45). However, some animal studies have suggested that folate status may have the opposite effect on intestinal tumorigenesis depending on the timing and dose of folate intervention (4–8). The contradicting effect of dietary folate on mammary and intestinal tumorigenesis in animal models using the same diets suggests that folate may modulate carcinogenesis in a tissue- and/or carcinogen-specific manner. The results from the present study are, however, consistent with those of a previous study that investigated the effect of dietary folate deficiency and supplementation on initiation and early promotion of MNU-induced mammary tumorigenesis in Fischer 344 rats (25). Baggott and colleagues performed a study in which rats were fed a casein-based AIN-76A diet containing either 0, 2 or 40 mg folic acid/kg diet, or 20 mg folinic acid/kg diet at weaning (27 days of age) for 30 days, injected with MNU intravenously (50 mg/kg body wt), and subsequently fed the control diet containing 2 mg folic acid/kg for 180 days. Glycine and succinylsulfathiazole (10 g/kg diet) were added to the diet to potentiate folate deficiency. Plasma folate concentrations were 15 ± 5, 77 ± 15 and 218 ± 47 ng/ml for the 0, 2 and 40 mg folic acid/kg diet groups at the time of MNU injection and 79 ± 8, 58 ± 6 and 56 ± 6 ng/ml at necropsy. Although the incidence of mammary cancer was not significantly different among the four groups,

cancer multiplicity was significantly lower in rats fed the 0 mg folic acid diet than those fed the 2 mg folic acid, the 40 mg folic acid or the 20 mg folinic acid diets; there was no significant difference in cancer multiplicity among the latter three groups. Furthermore, the time required for 50% of the rats to develop palpable mammary tumors was significantly longer in the 0 mg folic acid group than in the 40 mg folic acid or the 20 mg folinic acid group, but was not significantly different from that in the 2 mg folic acid group. Thus, Baggott's study demonstrated that folate deficiency suppressed initiation and early promotion of MNU-induced mammary tumorigenesis (25).

As suggested by Baggott's study (25), the inhibitory effect of folate deficiency on MNU-induced mammary tumorigenesis in rats may be a real effect on initiation and early promotion. However, it is possible that the conventional dose and route of MNU injection employed in the present study might have created an overwhelmingly carcinogenic milieu for folate status to modulate initiation of mammary tumorigenesis. Regardless of the levels of dietary folate, MNU probably induced and established neoplastic foci in mammary tissues. In this setting, folate deficiency probably suppressed the progression of and/or caused regression of established mammary neoplastic foci. This explanation is consistent with the biochemical function of folate. Interruption of folate metabolism in rapidly replicating neoplastic cells to cause ineffective DNA synthesis and hence the inhibition of tumor growth has been the basis of antitumor therapy using antifolate agents (49). Folate deficiency has been shown to induce regression and suppress progression of pre-existing neoplasms in experimental models (4,7,8,50–52). Therefore, it is possible that the inhibitory effect of folate deficiency on MNU-induced tumorigenesis in this rat model might have been primarily on promotion/progression of established mammary neoplastic foci. In this regard, although Baggott's study was primarily designed to test the effect of folate on initiation and early promotion, it is possible that the observed effect of folate was actually on promotion/progression because of the dose and route of MNU employed in that study (25).

In the present study, dietary folate supplementation at four times the basal dietary requirement, which has consistently conferred protection against intestinal tumorigenesis in rodents in previous studies (4,7,8,27), did not inhibit mammary tumorigenesis. This level of dietary folate supplementation did not promote the progression of MNU-induced mammary neoplastic foci in the present study in contrast to the promoting effect associated with this level of dietary folate supplementation on progression of established intestinal neoplastic foci observed in some studies (7,8). The lack of effect associated with folate supplementation on mammary tumorigenesis in the present study may be related to the fact that, in spite of significantly higher serum and hepatic folate levels, the mean mammary gland folate concentration of the folate-supplemented rats was not significantly different from that of the controls. Previous studies have demonstrated a dose–responsive tissue saturating effect of folate supplementation above four times the basal dietary requirement in rat colon (4), and the 8 mg folic acid diet has consistently induced significantly higher colonic mucosal folate concentrations compared with the 2 mg folic acid (control) diet in rodents (4,7,8,46,53). It is well known that different tissues express different folate requirements and hence different susceptibility to folate deficiency (40). Furthermore, folate accumulation in tissues is limited by the level of FPGS activity in the setting of substrate excess (47,48). FPGS catalyzes polyglutamation of intracellular folates, thereby allowing the retention of folate that would otherwise be lost because of efflux from the cell (47,48). Previous studies in animals and in cultured cells have shown that tissue levels of folate reach a plateau when FPGS is saturated from excess folate in the diet or culture medium (4,47,48). At present, there is no information in the literature regarding the levels of FPGS activity in normal mammary tissue. It is possible that the levels of FPGS activity in mammary gland are appreciably lower than the liver or colon and thus tissue folate is saturated at a much lower level of dietary folate in mammary gland compared with other tissues. However, it is also possible that higher levels of dietary folate supplementation above four times the basal dietary requirement may be necessary to increase mammary folate concentrations compared with the control diet.

One interesting finding in this study is that the extent of genomic DNA methylation is significantly lower in mammary adenocarcinomas than in non-neoplastic mammary tissues regardless of folate status. DNA methylation is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromatin modifications and in the development of mutations (29,30). Neoplastic cells simultaneously harbor widespread genomic DNA hypomethylation and more specific regional areas of hypermethylation (29,30). Genomic hypomethylation is an early, and consistent, event in carcinogenesis and is associated with genomic instability and increased mutations (29,30). Site-specific hypomethylation at the promoter region of tumor suppressor and mismatch repair genes is an important mechanism in gene silencing in carcinogenesis (29,30). Although promoter CpG islands hypermethylation and consequent inactivation of several tumor suppressor genes have been observed in human breast cancer (54), very few studies have reported genomic hypomethylation in human breast cancer (55,56). To our knowledge, our study is the first to demonstrate that genomic DNA hypomethylation is an epigenetic phenomenon associated with MNU-induced mammary tumorigenesis in rats. The extent of genomic DNA methylation in mammary adenocarcinomas and in non-neoplastic mammary tissues was not significantly modulated by folate status. This observation suggests that altered genomic DNA methylation was not a probable mechanism by which folate deficiency suppressed mammary tumorigenesis in our study. Because folate may modulate DNA methylation in a site-specific manner (43), however, the possibility that folate status may affect site-specific methylation of critical genes implicated in mammary tumorigenesis cannot be ruled out in the present study.

The strengths of the present study include: (i) the use of the amino acid-defined diet that is widely accepted as the standard means of inducing folate deficiency or providing supplemental dietary folate in rodents; (ii) the use of dietary levels of folate that have been shown to modulate development of other cancers in this strain of rats; (iii) measurements of systemic and mammary gland folate concentrations; (iv) rigorous histological confirmation of all mammary tumors to ensure an accurate determination of the rate of appearance and other tumor-specific parameters of adenomas and adenocarcinomas. However, several limitations associated with the present study need to be acknowledged. First, although the dose and route of MNU administration employed in the present study may be appropriate in studies examining the effect of other potential

chemopreventive agents in this model, the effect may be too overwhelmingly carcinogenic for folate to modulate. Therefore, the effect observed with dietary folate in the present study may be predominantly on promotion and progression, and not on initiation, of MNU-induced neoplastic foci. Secondly, the fat content of the diets used in the present study was higher than the AIN rodent diets that are more commonly used in experimental mammary tumor studies (10 versus 7% by weight). Animal studies have generally suggested that high fat diets enhance mammary tumorigenesis in rodents (57). Therefore, it is possible that the tumor-promoting effect associated with the higher fat content in our diets might have masked any modulating effect of dietary folate intervention. Thirdly, the mean mammary gland folate concentration associated with folate supplementation was not significantly higher than that of the control diet. Therefore, higher levels of folate supplementation above four times the basal dietary requirement may be necessary to significantly increase mammary gland folate concentrations and to observe any modulatory effect of folate supplementation on mammary tumorigenesis. Lastly, the number of animals employed in the present study did not allow us to achieve adequate statistical power. It would have required 103 animals in total to be 80% certain of detecting a 35% reduction in tumor incidence associated with folate deficiency compared with the control diet at a 5% level of significance.

In summary, our data suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats. In contrast, dietary folate supplementation at four times the basal dietary requirement does not significantly modulate mammary tumorigenesis. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammary tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer.

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# Effects of dietary folate on the development and progression of mammary tumors in rats[1]

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3. The abbreviations used are: CpG, cytosine-guanine dinucleotides; FPGS, folylpolyglutamate synthetase; MTHFR, methylenetetrahydrofolate reductase; MNU, *N*-methyl-*N*-nitrosourea; and SAM, S-adenosylmethionine.

## ABSTRACT

Epidemiologic studies have suggested that dietary intake and blood levels of folate may be inversely related to the risk of breast cancer. However, epidemiologic evidence has not been consistent nor has it provided unequivocal support for this purported inverse relationship. Recent evidence has also raised a concern that folate supplementation may promote carcinogenesis if provided after neoplastic foci are established in the target organ. This study investigated the effect of dietary folate deficiency and supplementation on the develpment and progression of mammary tumors in the N-methyl-N-nitrosourea (MNU) rat model. Weanling, female Sprague-Dawley rats were fed diets containing either 0 mg (moderate deficiency), 2 mg (basal dietary requirement, control) or 8 mg (supplementation) folic acid/kg diet during the initiation or promotion phase of MNU-induced mammary tumorigenesis. At necropsy, all macroscopic mammary tumors were identified and histologically confirmed. Dietary folate deficiency and supplementation provided during the initiation phase did not significantly modulate the development of mammary tumors. In contrast, dietary folate deficiency provided during the promotion phase significanlty inhibited the rate of appearance, incidence, mean volume and weight of adenocarcinomas compared with the control and supplemental diets. Folate supplementation provided during the promotion phase did not significantly modulate mammary tumorigenesis compared with the control group. Notwithstanding the limitations associated with this model, our data, in conjunction with the portfolio of epidemiologic evidence, do not support the purported inverse association between folate status and the risk of breast cancer. The use of folic acid supplementation to prevent breast cancer should not be recommended at present.

## INTRODUCTION

Dietary intake and blood levels of folate, a water-soluble B-vitamin and important co-factor in one carbon metabolism (1), appear to be inversely associated with the risk of several malignancies including cancer of the colorectum, lungs, pancreas, esophagus, stomach, cervix, and ovary, and neuroblastoma and leukemia (2, 3). The precise nature and magnitude of the relationship between folate status and the risk of these malignancies, however, are not uniformly consistent (2, 3). As an essential co-factor for the de novo biosynthesis of purines and thymidylate, folate plays an important role in DNA synthesis, stability and integrity, and repair, aberrations of which have been implicated in carcinogenesis (4, 5). Folate, in the form of 5-methyltetrahydrofolate, is also involved in remethylation of homocysteine to methionine, which is a precursor of S-adenosylmethionine, the primary methyl group donor for most biological methylations including that of DNA (6). In this role, folate may modulate DNA methylation, which is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromosomal modifications, and in the development of mutations (7). A growing body of evidence from in vitro, animal, and human studies indicates that folate deficiency is associated with DNA strand breaks, impaired DNA repair, increased mutations, and aberrant DNA methylation, and that folate supplementation can correct some of these defects induced by folate deficiency (2, 4-6, 8).

An accumulating body of epidemiologic studies also suggest an inverse association between folate status and the risk of breast cancer (9-22). However, epidemiologic evidence available thus far has not been consistent nor has it provided unequivocal support for the purported inverse relationship between folate status and breast cancer risk (23-28). Some epidemiologic studies have suggested that folate status alone may not be sufficient in modifying

breast cancer risk. However, with alcohol consumption, a well-established risk factor for breast cancer development (29, 30), folate deficiency potentiates, whereas folate supplementation reduces, the risk of breast cancer (12, 14, 15, 19, 20, 22). Furthermore, some sudies have suggested that folate status may modify breast cancer risk in conjunction with other dietary factors involved in one-carbon metabolism such as methionine, vitamins $B_6$ and $B_{12}$ (15, 19). Also, there is evidence that the direction and magnitude of the breast cancer risk modification associated with folate status may depend on the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene that encodes an enzyme critical for intracellular folate homeostasis (16, 31-34).

Because only few modifiable risk factors for breast cancer exist (30, 35), recent epidemiologic observations that suggest that folate deficiency increases, whereas supplementation reduces, the risk of breast cancer underscore the need to further evaluate the role of folate in the development of this disease. Folate is an ideal agent for potential chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects (36), and possesses biologically plausible mechanisms for cancer prevention (2, 4-6, 8, 37). However, studies performed in animal models of colorectal cancer have shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels and folate intervention after microscopic neoplasia are established in the colorectal mucosa promote rather than suppress colorectal carcinogenesis (38). These observations suggest that folate possesses the dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention (38). Folate deficiency has an inhibitory effect whereas folate supplementation has a promoting effect on the progression of established neoplasms (38). In contrast, folate deficiency in normal epithelial

tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress, whereas supraphysiological doses enhance, the development of tumors in normal tissues (38).

The dual modulatory role of folate in carcinogenesis underscores the importance of clarifying the potential role of folate in breast cancer prevention in appropriate animal models before folate supplementation can be considered in humans. In this regard, three published animal studies have produced conflicting results. In mice with confirmed spontaneous mammary cancer, daily intravenous injections of fermentation *Lactobacillus casei* factor (pteroyltriglutamate) significantly regressed pre-existing mammary tumors and decreased new mammary tumor formation and lung metastases (39). In contrast, another study employing the *N*-methyl-*N*-nitrosourea (MNU) rat model showed that a folate-deficient diet provided during the initiation phase of mammary tumorigenesis significantly reduced tumor multiplicity and increased tumor latency, but had no effect on tumor incidence, compared with a control and folate-supplemented diet (40). We have recently reported that dietary folate deficiency of a moderate degree suppresses, whereas a modest level of folate supplementation does not significantly modulate, mammary tumorigenesis in the MNU rat model (41). Given these considerations, the aim of this study was to elucidate the effects of dietary folate deficiency and supplementation on the development and progression of mammary tumors by determining the effects of folate on the initiation and promotion phases of mammary tumorigenesis separately in the well-established MNU rat model of breast cancer. Our hypothesis was that dietary folate deficiency would enhance, whereas dietary folate supplementation would suppress, the initiation of MNU-induced mammary tumorigenesis. In contrast, once microscopic mammary neoplastic foci are established, we reasoned that dietary folate deficiency would suppress, whereas dietary

folate supplementation would enhance, the promotion and progression of MNU-induced mammary tumorigenesis.

## MATERIALS and METHODS

### Animals and dietary intervention

This study was approved by the Animal Care Committee of the University of Toronto. Specific pathogen-free, weanling female Sprague-Dawley rats (~50 g; Charles River Laboratories, St. Constant, Quebec, Canada) were singly housed and maintained at 24 ± 2°C at 50% humidity with a 12 h light/dark cycle. Amino acid- defined diets (Dyets, Bethlehem, PA) (42) containing either 0, 2 or 8 mg folic acid/kg diet were used. These diets constitute a standard method of inducing folate deficiency or providing supplemental dietary folate in rodents (42) and have been utilized extensively in previous studies of folate and colorectal cancer (43-46) and breast cancer (41). The diet containing 0 mg folic acid/kg produces progressive folate deficiency of a moderate degree without anemia, growth retardation or premature death through weeks 3-5, after which systemic folate indicators stabilize (41, 43). Although this diet is completely devoid of folate, severe folate deficiency is not induced because of de novo synthesis of folate by intestinal bacteria, some of which is incorporated into the tissue folate of the host (47). Two mg folic acid/kg diet is generally accepted as the basal dietary requirement for rodents (48). The diet containing 8 mg folic acid/kg represents folate supplementation 4x the basal dietary requirement. This level of folate was chosen because the 8 mg/kg level has consistently provided a degree of chemoprevention against colorectal cancer in previous rodent studies (43-45). These diets contained 50 g cellulose/kg, 60% of the calories as carbohydrates, 23% as fat (or 10% by weight), and 17% as L-amino acids (42). The amount of methyl donors, methionine, choline, and vitamin $B_{12}$, 8.2 g, 2.0 g, and 50 $\mu$g per kg diet, respectively. The detailed composition of the diets has been published previously (42, 46). Diets and water were provided ad libitum.

In the initiation study, rats (n=61) were randomized to receive the diet containing either 0 (n=21), 2 (n=20), or 8 (n=20) mg folic acid/kg diet from weaning at 3 weeks of age for 5 weeks until one week following MNU injection. At 50 days of age, all the rats received an i.p. injection of MNU. The initial diets were terminated one week after the MNU injection, and all the rats were placed on the control diet (2 mg folic acid/kg diet) for 22 weeks until the time of sacrifice.

In the promotion study, rats (n=93) were placed on the control diet (2 mg folic acid/kg diet) from weaning at 3 weeks of age for 5 weeks until one week following MNU injection. At 50 days of age, all the rats received an i.p. injection of MNU. One week following MNU administration, the rats were randomized to receive the diet containing either 0 (n=33), 2 (n=30), or 8 (n=30) mg folic acid/kg diet for 22 weeks until the time of sacrifice.

**MNU administration**

Notwithstanding the limitations associated with animal models, the MNU rat model is widely used to determine the effects of dietary factors on mammary tumorigenesis for the following reasons: (a) histological similarities of adenocarcinoma to human breast cancer; (b) local invasiveness and metastatic potential; (c) a clear operational distinction between the initiation and promotion stages; and (d) hormonally dependent mammary tumorigenesis (49-53). At 50 days of age, all rats received one i.p. injection of MNU (50 mg/kg body weight; Sigma Chemical Co., St. Louis, MO). A single injection of 50 mg MNU/kg has become the standard dosage due to its rapid induction and high incidence of mammary tumors combined with minimal toxicity and a short latency period of 3-6 months (49, 50).

**Observation parameters**

Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week. All rats were palpated for mammary tumors once a week beginning 4 weeks after MNU administration. The number, size and location of each tumor were recorded in a manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All the rats were monitored daily for clinical evidence of illness or morbidity and those approaching a moribund state were promptly killed. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15-20 mm in diameter, tumors that impaired normal movement of the animals, and ulcerating tumors were immediately killed during the study.

**Sample collection and analysis of mammary tumors**

Blood was withdrawn from the lateral tail vein of each rat within a week of MNU injection and from the heart at necropsy and centrifuged at 5000 rpm for 10 min at 4°C. Serum was stored at -70°C in 0.5% ascorbic acid for serum folate assay. Given the latency period of 3-6 months associated with a single i.p. MNU injection and the average duration for the systemic and tissue folate levels to stabilize, the rats were killed by carbon dioxide inhalation followed by cervical dislocation at 23 weeks after MNU injection (30 weeks of age). The liver from each rat was harvested, snap-frozen and stored at -70°C for determination of hepatic folate concentration. All macroscopic mammary tumors were counted, excised and weighed, and the diameter of each tumor was measured using a digital caliper for final tumor volume computation in a blinded fashion. One-half of each macroscopic tumor was snap-frozen in liquid nitrogen and stored at -70°C for DNA extraction. The other half of the tumor was fixed in 10% neutral-buffered formalin, processed in a standard manner for hematoxylin-eosin (H&E) staining and

histologically analyzed according to Russo et al. (52) by two experienced pathologists (A.M. and R.R.) blinded to the study group independently. Normal mammary tissue was also excised at necropsy from each rat, snap-frozen in liquid nitrogen and stored at -70°C for DNA extraction and mammary tissue folate determination.

**Determination of folate concentration**

Serum folate concentrations were determined by a standard microbiological microtiter plate assay using *Lactobacillus casei* (54). Hepatic and normal mammary tissue folate concentrations were measured by the same microbiologic assay (54), utilizing a previously described method for the determination of tissue folates (55).

**DNA extraction**

DNA from normal mammary tissue and mammary tumors was extracted by standard technique using a lysis buffer containing proteinase K followed by phenol, chloroform, and isoamyl alcohol organic extraction (56). The size of DNA estimated by agarose-gel electrophoresis was >20 kb in all instances. No RNA contamination was detected on agarose-gel electrophoresis. The final preparations had a ratio of $A_{260}$ to $A_{280}$ between 1.8 and 2.0. The concentration of each DNA sample was determined as the mean of 3 independent spectrophotometric readings.

**Genomic DNA methylation determination**

The methylation status of cytosine-guanine (CpG) sites in genomic DNA from normal mammary tissue and mammary tumors was determined by the in vitro methyl acceptance capacity of DNA using $^{3}$H-methyl-SAM as a methyl donor and a prokaryotic CpG DNA methyltransferase, Sss1, as previously described (41, 44, 46, 57, 58). The manner in which this

assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous $^{3}$H-methyl incorporation. All analyses were performed in duplicate.

**Statistical Analysis**

Between-group comparisons of continuous variables were assessed using the Kruskal-Wallis and Mann-Whitney non-parameteric tests. For categorical response variables, differences among the groups were assessed by Pearson $\chi^2$ test. Differences in genomic DNA methylation between normal mammary gland and tumor in each diet group was assessed by the Wilcoxon signed ranks test. The Kaplan-Meier survival analysis and the Log Rank test were used to compare the rates of tumor appearance among the three groups. All significance tests were two sided and were considered statistically significant if the observed significance level was $<0.05$. Results are expressed as mean ± SEM. Statistical analyses were performed using SPSS (version 10).

## RESULTS

### Body weight and daily food consumption

Growth curves were not significantly different among the three dietary groups in both the initiation and promotion studies; at no time point did the mean body weights differ significantly among the three groups (data not shown). This finding indicates that folate deficiency in the rats fed 0 mg folate/kg diet was moderate; otherwise, growth retardation or premature death would have occurred (59). The mean daily food consumption, which was determined on a pre-assigned day of each week, was not significantly different among the three groups in both the initiation and promotion studies.

### Serum, liver and normal mammary gland folate concentrations

*Initiation study*: At the time of MNU injection (4 weeks after the start of dietary intervention) the mean serum folate concentrations were significantly different among the three groups ($P<0.001$; Table 1). The mean serum folate concentrations of the three dietary groups at this time point were comparable to those observed in rats placed on the corresponding diets for 4 – 5 weeks in previous studies (41, 43, 44). This observation indicates that a sufficient degree of systemic folate deficiency and supplementation was achieved in the folate-deficient and supplemented rats, respectively, at the time of MNU injection for the determination of the effect of folate status on the initiation phase of MNU-induced mammary tumorigenesis. At necropsy (22 weeks after placed on the control diet), the mean serum, hepatic, and mammary gland folate concentrations of the three groups were not significantly different (Table 1), and these levels were comparable with those observed in rats placed on the same diet for 24 – 27 weeks in previous studies (41, 43, 60).

*Promotion study*: At the time of MNU injection (4 weeks after placed on the control diet) the mean serum folate concentrations were not significantly different among the three groups (Table 1). The mean serum folate concentrations of the three dietary groups at this time point were comparable to those observed in rats placed on the same diets for 4 – 5 weeks in previous studies (41, 43, 44). At necropsy (22 weeks after the start of dietary intervention), the mean serum and hepatic folate concentrations were significantly different among the three dietary groups ($P<0.001$; Table 1) and were comparable to those observed in rats placed on the corresponding diets for 24 – 27 weeks in previous studies (41, 43, 44). This observation indicates that a sufficient degree of systemic folate deficiency and supplementation was induced in the folate-deficient and supplemented rats, respectively, after MNU injection for the determination of the effect of folate status on the promotion phase of MNU-induced mammary tumorigenesis. The mean mammary gland folate concentration of the folate-deficient group was significantly lower than the control and folate-supplemented groups ($P\leq0.004$; Table 1). Although the mean mammary gland folate concentration of the folate-supplemented group was higher than that of the control group, this difference did not reach statistical significance ($P=0.063$; Table 1).

**Effects of dietary folate on MNU-induced mammary tumorigenesis**

No rats died prematurely or were killed before necropsy in the three dietary groups for reasons other than the presence of large and/or ulcerating tumors as defined in the Materials and Methods section. The prevalence of euthanized rats was similar among the three groups. Consistent with previous observations made in the MNU-Sprague-Dawley rat model of mammary tumorigenesis (49-53), >90% of macroscopic mammary tumors in the present study were identified histologically as either adenomas (15%) or adenocarcinomas (85%). There was

an excellent agreement in histological diagnosis of either adenoma or adenocarcimona between the two study pathologists (kappa statistic = 0.95). The analyses pertaining to mammary tumors were performed for the combination of adenocarcinomas and adenomas and for adenocarcinomas alone. There were not a sufficient number of adenomas for independent analysis.

*Initiation study*: The rates of appearance of either adenocarcinomas or adenomas among the three dietary groups were not significantly different (P=0.83; Figure 1A). When the analysis was confined to adenocarcinomas alone, no significant difference was observed among the three groups (P=0.81; Figure 1B). As shown in Table 2, there was no significant difference in the final incidence, mean tumor latency (mean time to appearance of first palpable tumor), multiplicity (mean number of tumors per tumor-bearing rat), volume or weight of adenocarinomas and adenomas at necropsy among the three groups. A similar observation was made when the analyses were confined to adenocarcinomas alone (Table 2).

*Promotion study*: There was a non-significant trend towards a difference in the rates of appearance of either adenocarcinomas or adenomas among the three dietary groups (P=0.10) with the folate-deficient group demonstrating a trend towards a decrease in the rates compared with the conrol and folate-supplemented groups (Figure 2A). When the analysis was confined to adenocarcinomas alone, a significant difference in the rates of appearance of adenocarcinomas among the three groups was observed (P=0.02; Figure 2B). The rate of appearance of adenocarcinomas in the folate-deficient group was significantly lower than those in the control (P=0.01) and folate-supplemented (P=0.02) groups (Figure 2B). There was no significant difference between the control and folate-supplemented groups (P=0.87) (Figure 2B).

There was a non-significant trend towards lower final incidence and multiplicity of adenocarcinomas and adenomas at necropsy in the folate-deficient group compared with the control and folate-supplemented groups (P=0.18 and P=0.13, respectively; Table 3). The mean tumor volume of adenocarcinomas and adenomas in the folate-deficient group was significantly lower than that of the control and folate-supplemented groups (P<0.02) whereas no difference was observed between the control and folate-supplemented groups (Table 3). The mean tumor weight of the adenocarcinomas and adenomas in the folate-deficient group was nonsignificantly and significantly lower than that of the control (P=0.18) and folate-supplemented (P=0.005) groups, respectively, whereas no significant difference was observed between the control and folate-supplemented groups (Table 3). The mean tumor latency was not significantly different among the three groups (Table 3). When the analyses were confined to adenocarcinomas alone, a similar pattern was observed (Table 3) and a clearer picture emerged with respect to the final incidence of adenocarcinomas; the final incidence of adenocarcinomas was significantly lower in the folate-deficient group than in the control and folate-supplemented groups (P<0.04) whereas no significant difference was observed between the control and folate-supplemented groups (Table 3).

**Effect of dietary folate on genomic DNA methylation in mammary tumors and non-neoplastic mammary tissues**

Given the role of folate in DNA methylation (6), an important epigenetic determinant in carcinogenesis (7), we investigated whether dietary folate modulates genomic DNA methylation in MNU-induced mammary tumorigenesis. For both the initiation (Figure 3A) and promotion (Figure 3B) studies, the degree of $^{3}$H-methyl incorporation into DNA of the mammary

adenocarcinoma and into DNA from the pair-matched non-neoplastic mammary tissue was not significantly different among the three dietary groups. However, the degree of $^{3}$H-methyl incorporation into DNA of the mammary adenocarcinomas, which is inversely related to the extent of genomic DNA methylation, was 3- to 5-fold higher than that of non-neoplastic mammary tissue within each dietary group ($P<0.04$; Figures 3A and 3B), indicating a significantly lower degree of genomic DNA methylation in adenocarcinomas compared with normal mammary tissue.

## DISCUSSION

Our data from the initiation study indicate that dietary folate deficiency and supplementation do not significantly modulate the development of MNU-induced mammary tumors in this rodent model. This observation suggests that the conventional dose and route of MNU administration in this study created an overwhelmingly carcinogenic milieu for folate status to modulate the initiation of mammary tumorigenesis. Regardless of the levels of dietary folate, MNU induced and established neoplastic foci in mammary tissues. The lack of effect of dietary folate on the initiation of MNU-induced mammary tumorigenesis is consistent with prior epidemiologic observations that have suggested that folate status alone may not be sufficient to modulate the development of breast cancer but only in conjunction with alcohol (12, 14, 15, 19, 20, 22), with other folate co-factors (12, 14, 15), or with genetic predispositions (e.g., MTHFR C677T polymorphism) (16, 31-34). This is also in keeping with the similar observations pertaining to the role of folate in colorectal (43) and uterine cervical (61) carcinogenesis.

After neoplastic foci are established with MNU administration, however, our data from the promotion study clearly indicate that dietary folate deficiency of a moderate degree significantly suppressed the progression of or caused regression of established mammary neoplastic foci in rats. This explanation is supported by the observed significant inhibitory effect of folate deficiency on the rate of appearance, final incidence, mean volume and weight of mammary tumors as well as a trend towards decreased multiplicity of tumors in the promotion study. Several prior observations made in animal models support the inhibitory effect of folate deficiency on established or existing neoplastic foci. In $Apc^{Min}$ and *Apc+/-Msh2-/-* murine models of intestinal tumorigenesis, if folate intervention was started before the establishment of neoplastic foci in the intestine, moderate folate deficiency enhanced, whereas modest levels (4 –

10 times the basal dietary requirement) of folate supplementation suppressed, the development of intestinal tumors (45, 46). If, however, folate intervention was started after the establishment of neoplastic foci, dietary folate had an opposite effect on the progression of intestinal tumors (45, 46). Folate deficiency has also been shown to induce regression and suppress progression of pre-existing neoplasms in experimental models (62-64). The inhibtory effect of folate deficiency on established neoplastic foci is also consistent with the known biochemical function of folate. As an essential co-factor for the de novo biosynthesis of purines and thymidylate, folate plays an important role in DNA synthesis and replication (1). Folate deficiency in tissues with rapidly replicating cells results in ineffective DNA synthesis. In neoplastic cells where DNA replication and cell division are occurring at an accelerated rate, interruption of folate metabolism causes ineffective DNA synthesis, resulting in inhibition of tumor growth. This has been the basis for cancer chemotherapy using anitfolate agents (65).

We hypothesized that once neoplastic foci are established with MNU administration, folate supplementation would enhance the promotion and progression of MNU-induced mammary tumorigenesis. This was based on the observed promoting effect of folate supplementation on established intestinal neoplastic foci in $Apc^{Min}$ and *Apc+/-Msh-/-* mice (45, 46) and on the previously reported accelerated progression of leukemia in children with acute leukemia treated with folate supplementation (66). In the present study, dietary folate supplementation at 4x the basal dietary requirement, which has previously shown to promote established intestinal neoplastic foci (45, 46), did not significantly promote the progression of MNU-induced mammary neoplastic foci. This lack of promoting effect associated with folate supplementation on established mammary neoplastic foci in the present study may be a real tissue-specific observation limited to mammary tumors. Another possible explanation is related

to the fact that, in spite of significantly higher serum and hepatic folate levels, the mean mammary gland folate concentration of the folate-supplemented rats was not significantly different from that of the controls. Previous studies have demonstrated a dose-responsive tissue saturating effect of folate supplementation above 4x the basal dietary requirement in rat colon (44), and the 8 mg folic acid diet has consistently induced signficantly higher colonic mucosal folate concentrations compared with the 2 mg folic acid (control) diet in rodents (44-46, 60, 67). However, it is well known that different tissues express different folate requirements and hence different susceptibility to folate deficiency (55). Furthermore, the expression and activity of folylpolyglutamyl synthetase (FPGS) and γ-glutamyl hydrolase (GGH), enzymes responsible for intracellular folate retention and efflux, respectively, are significantly different among different tissues (1). Folate accumulation in tissues is limited by the level of FPGS activity in the setting of substrate excess (1, 68). Previous studies in animals and in cultured cells have shown that tissue levels of folate reach a plateau when FPGS is saturated from excess folate in the diet or culture medium (1, 44, 68). It is possible that the levels of FPGS activity in mammary gland are appreciably lower than the colon and thus tissue folate is saturated at a much lower level of dietary folate in mammary gland compared with other tissues. However, it is also possible that higher levels of dietary folate supplementation above 4x the basal dietary requirement may be necessary to increase mammary folate concentrations compared with the control diet in order to exert any effect on tumor progression.

Our data corroborate the findings from our previous work that demonstrated that dietary folate deficency of moderate degree provided from weanling through MNU injection for 27 weeks signifincantly inhibited, whereas dietary folate supplementation 4x the basal requirement did not modulate, mammary tumorigenesis in this rat model (41). Our data clearly indicate that

the inhibitory effect of folate deficinecy on MNU-induced mammary tumorigenesis in this rat model is primarily on promotion and progression of established mammary neoplastic foci. The lack of effect associated with dietary folate deficiency and supplementation on the initiation of MNU-induced mammary tumorigenesis in our study, however, differ from that of a previous study that has demonstrated an inhibitory effect of folate deficiency on both the initiation and early promotion phases of MNU-induced mammary tumorigenesis in rats (40). Baggott and colleagues performed a study in which Fischer 344 rats were fed a casein-based AIN-76A diet containing either 0, 2 or 40 mg folic acid/kg diet, or 20 mg folinic acid/kg diet at weaning (27 days of age) for 30 days, injected with MNU intravenously (50 mg/kg body weight), and subsequently fed the control diet containing 2 mg folic acid/kg for 180 days. Although the incidence of mammary cancer was not significantly different among the 4 groups, cancer multiplicity was significantly lower in rats fed the 0 mg folic acid diet than those fed the 2 mg folic acid, the 40 mg folic acid or the 20 mg folinic acid diets; there was no signficant difference in cancer multiplicity among the latter 3 groups. Furthermore, the time required for 50% of the rats to develop palpable mammary tumors was significantly longer in the 0 mg folic acid group than in the 40 mg folic acid or the 20 mg folinic acid group, but was not significantly different from that in the 2 mg folic acid group. Baggot's study is significantly different from our study in several important aspects including the use of non-standard dietary means to modulate folate status, possible growth retardation of animals, the concomitant use of antibiotics that may independently affect folate levels, and the use of animals that are resistant to chemically-induced mammary tumorigenesis. Furthermore, although Baggott's study was primarily designed to test the effect of folate on initiation and early promotion, it is possible that the observed effect of

folate was actually on promotion/progression because of the dose and route of MNU employed in that study.

Three animal studies (40, 41), including the present study, all conducted in the standard MNU rodent model of mammary tumorigenesis, collectively suggest that dietary folate deficiency inhibits, whereas folate supplementation (4x – 20x above the basal dietary requirement) does not significantly modulate, the development and progression of mammary tumorigenesis. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests an inverse association between folate status and the risk of breast cancer. However, epidemiologic evidence available thus far has not been consistent nor has it provided unequivocal support for this purported inverse relationship. Among 13 published case-control studies that investigated the relationship between dietary folate intake and breast cancer risk, 10 showed either a significant or equivocal inverse relationship that was not statistically significant, that became nonsignificant after adjustment, or that could not be distinguished from other factors in their relation to risk (9-18), whereas 3 showed an unequivocal null association (23-25). In some studies, the observed inverse association was further modified by the intake of alcohol and other folate cofactors (e.g. methionine, vitamins $B_6$ and $B_{12}$) (12, 14, 15). Two large prospective studies (the Nurses' Health Study and the Iowa Women's Heath Study) have shown a weak inverse association between the total or dietary intake of folate and breast cancer risk (19, 20). These studies, however, have indicated that low intakes of folate increase, whereas high intakes of folate decrease, breast cancer risk among women who regularly consume alcohol (19, 20), providing further evidence for folate-alcohol interactions in breast carcinogenesis observed in case-control studies (12, 14, 15). The Iowa Women's Health Study further demonstrated that the increased risk of breast cancer associated with low folate and high

alcohol intake was limited to estrogen receptor (ER) negative breast cancer (21). However, two other large prospective studies (the American Cancer Society Cancer Prevention Study II Nutrition Cohort and the Nurses' Health Study II) found no association between the risk of breast cancer and dietary or total folate (26, 27) and no evidence of an interaction between folate and alcohol in modifying breast cancer risk (27). With respect to actual blood levels of folate and breast cancer risk, one nested case-control study, using stored serum samples from the Washington County (Maryland) serum bank, found no association between serum folate levels and breast cancer risk (28), whereas another nested case-control study (the Nurses' Health Study) demonstrated a weak inverse association between plasma folate levels and breast cancer risk, which was significantly modified by alcohol consumption (22). In these studies, blood levels of vitamins $B_6$ and $B_{12}$ appeared to be inversely related to breast cancer risk (22, 28). Recent molecular epidemiologic studies have shown that the MTHFR C677T polymorphism may modulate breast cancer risk, albeit not uniformly consistent, and that the direction and magnitude of the risk modification may be influenced by folate status and alcohol consumption (16, 31-34). Overall, the portfolio of epidemiologic evidence supporting the relationship between folate status and breast cancer risk is tenuous at best, although a clearer picture emerges when studies examining the joint effects of folate and alcohol are considered.

One interesting finding in this study is that the extent of genomic DNA methylation is significantly lower in mammary adenocarcinomas than in nonneoplastic mammary tissues regardless of folate status. Neoplastic cells simultaneously harbor widespread genomic DNA hypomethylation and more specific regional areas of hypermethylation (7). Genomic hypomethylation is an early, and consistent, event in carcinogenesis and is associated with genomic instability and increased mutations (7). Site-specific hypermethylation at the promoter

region of tumor suppressor and mismatch repair genes is an important mechanism in gene silencing in carcinogenesis (7). Although promoter CpG islands hypermethylation and consequent inactivation of several tumor suppressor genes have been observed in human breast cancer (69), very few studies have reported genomic hypomethylation in human breast cancer (70, 71). Our study demonstrates that genomic DNA hypomethylation is an epigenetic phenomenon associated with MNU-induced mammary tumorigenesis in rats. The extent of genomic DNA methylation in mammary adenocarcinomas and in nonneoplastic mammary tissues was not significantly modulated by folate status, which is consistent with prior observations made in rodent liver and colon (6). This observation suggests that altered genomic DNA methylation was not a likely mechanism by which folate deficiency suppressed mammary tumorigenesis in our study. Because folate may modulate DNA methylation in a site-specific manner (6), however, the possibility that folate status may affect site-specific methylation of critical genes implicated in mammary tumorigenesis cannot be ruled out in the present study.

The strengths of the present study include: (i) the use of the amino acid-defined diet that is widely accepted as the standard means of inducing folate deficiency or providing supplemental dietary folate in rodents; (ii) the use of dietary levels of folate that have been shown to modulate development of other cancers in this strain of rats; (iii) measurements of systemic and mammary gland folate concentrations; and (iv) rigorous histological confirmation of all mammary tumors to ensure an accurate determination of the rate of appearance and other tumor-specific parameters of adenomas and adenocarcinomas. However, several limitations associated with the present study need to be acknowledged. First, although the dose and route of MNU administration employed in the present study may be appropriate in studies examining the effect of other potential chemopreventive agents in this model, the effect may be too overwhelmingly

carcinogenic for folate to modulate. Regardless of the levels of dietary folate, MNU likely induced and established neoplastic foci in mammary tissues. Therefore, only the effect of dietary folate on promotion and progression, but not on initiation, of MNU-induced neoplastic foci may be determined in this model. Second, the fat content of the diets used in the present study was higher than the AIN rodent diets that are more commonly used in experimental mammary tumor studies (10% versus 7% by weight). Animal studies have generally suggested that high fat diets enhance mammary tumorigenesis in rodents (72). Therefore, it is possible that the tumor-promoting effect associated with the higher fat content in our diets might have confounded the modulating effect of dietary folate intervention. Third, the mean mammary gland folate concentration associated with folate supplementation was not significantly higher than that of the control diet. Therefore, higher levels of folate supplementation above 4x the basal dietary requirement may be necessary to significantly increase mammary gland folate concentrations in order to observe any modulatory effect of folate supplementation.

In summary, our data suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats, and this effect appears to be primarily via inhibiton of the progression of established mammary neoplastic foci. In contrast, dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis in this model. Notwithstanding the limitations associated with this model, our data, in conjunction with the portfolio of epidemiologic evidence, suggest that the role of folate in mammaray tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer. In particular, interactions between folate and alcohol and between folate and other folate cofactors in modifying breast cancer risk merit further consideration. Also, the effect of genetic polymorphisms in the folate and alcohol metabolic

pathways on breast cancer risk and related gene-folate interactions in further modulating this effect need to be clearly elucidated. These studies will lead to more rational and logical strategies using folic acid to prevent breast cancer. For instance, individuals with the MTHFR 677TT genotype with inadequate folate intake or with significant alcohol consumption have been shown to have an increased risk of breast cancer (16, 31-34). These individuals may therefore benefit from targeted folic acid chemoprevention. Given the emerging body of evidence that suggests the dual modulatory role of folate on carcinogenesis depending on the timing and dose of folate intervention (38) and the lack of convincing evidence for the protective effect of folate supplementatin on breast cancer risk, however, the use of folic acid supplementation to prevent breast cancer should not be recommeded at present.

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## FIGURE LEGENDS

**Figure 1.** The rate of appearance of either mammary adenomas or adenocarcinomas (A) or adenocarcinomas alone (B) among the three dietary groups (P-overall=0.83 and P-overall=0.81, respectively, by the Kaplan-Meier survival analysis) in the initiation study. In the initiation study, groups I, II and III received the 0 (deficient), 2 (control) and 8 (supplemented) mg folic acid/kg diet, respectively, from weaning at 3 weeks of age for 5 weeks until one week following MNU injection (at 50 days of age), followed by the 2 mg folic acid/kg diet for 22 weeks until the time of sacrifice (30 weeks of age).

**Figure 2.** (A) The rate of appearance of either mammary adenomas or adenocarcinomas among the three dietary groups (P-overall =0.10 by the Kaplan-Meier survival analysis) in the promotion study. (B) The rate of appearance of mammary adenocarcinomas among the three dietary groups (P-overall=0.02 by the Kaplan-Meier survival analysis; P=0.01 between groups I and II, P=0.02 between groups I and III, and P=0.87 between groups II and III by the Log Rank test) in the promotion study. In the promotion study, groups I, II and III received the 2 mg folic acid/kg diet (control) from weaning at 3 weeks of age for 5 weeks until one week following MNU injection (at 50 days of age), followed by the 0 (deficient), 2 (control) and 8 (supplemented) mg folic acid/kg diet, respectively, for 22 weeks until the time of sacrifice (30 weeks of age).

**Figure 3:** Effects of dietary folate on genomic DNA methylation in mammary adenocarcinomas and non-neoplastic mammary tissues as determined by the in vitro methyl acceptance assay in the initiation (A) and promotion (B) studies. The manner in which this assay is performed

produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous [$^3$H-methyl] incorporation into DNA. * denotes significant differences by the Wilcoxon signed ranks test at $P<0.04$ compared with adenocarcinomas within each dietary group. Values are mean ± SD.

**Table 1: Serum, hepatic and mammary gland folate concentrations[1]**

| | | | | | | |
|---|---|---|---|---|---|---|
| **Initiation Study[2]** | | | | | | |
| | At the time of MNU injection (4 weeks of dietary intervention) | | | At necropsy (22 weeks of the 2 mg folic acid/kg diet) | | |
| **Diet Group (n)** | I (21) | II (20) | III (20) | I (21) | II (20) | III (20) |
| **Serum folate (ng/ml)** | 21.7±1.4[a] | 64.7±2.5[b] | 107.8±3.2[c] | 50.6±2.2 | 50.7±1.9 | 53.6±2.1 |
| **Hepatic folate (μg/g tissue)** | | | | 6.3±0.3 | 6.2±0.4 | 6.8±0.6 |
| **Mammary folate (ng/g tissue)** | | | | 125.2±10.6 | 141.0±18.5 | 129.9±10.2 |
| **Promotion study[3]** | | | | | | |
| | At the time of MNU injection (4 weeks of the 2 mg folic acid/kg diet) | | | At necropsy (22 weeks of dietary intervention) | | |
| **Diet Group (n)** | I (33) | II (30) | III (30) | I (33) | II (30) | III (30) |
| **Serum folate (ng/ml)** | 71.1±2.4 | 72.9±3.1 | 76.8±3.0 | 9.4±1.0[a] | 48.9±2.0[b] | 77.6±2.3[c] |
| **Hepatic folate (μg/g tissue** | | | | 2.7±0.4[a] | 7.3±0.3[b] | 9.3±0.3[c] |
| **Mammary folate (ng/g tissue)** | | | | 69.2±4.2[a] | 110.3±12.7[b] | 146.4±14.3[b] |

1. Results are expressed as mean ± SEM. Means in a row with different letters at each time point significantly differ at $P<0.005$ by between-group comparsions.
2. In the initiation study, rats were randomized to receive the diet containing either 0 (I, folate deficiency), 2 (II, control, basal dietary requirement), or 8 (III, supplemented) mg folic acid/kg diet from weaning at 3 weeks of age for 5 weeks until one week following MNU injection. At 50 days of age, all the rats received an i.p. injection of MNU. The initial diets were terminated one

week after the MNU injection, and all the rats were placed on the control diet (2 mg folic acid/kg diet) for 22 weeks until the time of sacrifice.

3. In the promotion study, rats were placed on the control diet (2 mg folic acid/kg diet) from weaning at 3 weeks of age for 5 weeks until one week following MNU injection. At 50 days of age, all the rats received an i.p. injection of MNU. One week following MNU administration, the rats were randomized to receive the diet containing either 0 (I, deficient), 2 (II, control), or 8 (III, supplemented) mg folic acid/kg diet for 22 weeks until the time of sacrifice.

**Table 2: Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary tumors in the initiation study**[1]

| | Adenocarcinomas + Adenomas | | | | Adenocarcinomas | | | |
|---|---|---|---|---|---|---|---|---|
| **Diet group**[2] | I | II | III | P-value, ANOVA | I | II | III | P-value ANOVA |
| **Incidence (%)** | 68 | 70 | 70 | 0.99 | 59 | 60 | 70 | 0.73 |
| **Mean latency (weeks post-MNU injection)** | 18.4±1.1 | 16.6±1.0 | 17.5±1.3 | 0.54 | 17.7±1.2 | 16.8±1.2 | 17.5±1.3 | 0.88 |
| **Mean multiplicity** | 1.6±0.3 | 3.2±1.8 | 1.1±0.1 | 0.52 | 1.3±0.2 | 3.6±2.2 | 1.1±0.1 | 0.39 |
| **Mean volume ($cm^3$)** | 4.0±1.6 | 4.2±1.8 | 2.0±0.9 | 0.50 | 5.5±2.1 | 3.8±1.8 | 2.0±0.9 | 0.23 |
| **Mean weight (g)** | 1.5±0.6 | 1.4±0.5 | 1.0±0.3 | 0.70 | 2.0±0.8 | 1.2±0.4 | 1.0±0.3 | 0.58 |

1. Results are expressed as mean ± SEM.
2. Groups I, II and III received the 0 (deficient), 2 (control) and 8 (supplemented) mg folic acid/kg diet, respectively, from weaning at 3 weeks of age for 5 weeks until one week following MNU injection (at 50 days of age), followed by the 2 mg folic acid/kg diet for 22 weeks until the time of sacrifice (30 weeks of age).

**Table 3: Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary tumors in the promotion study[1]**

| | Adenocarcinomas + Adenomas | | | | Adenocarcinomas | | | |
|---|---|---|---|---|---|---|---|---|
| **Diet group[2]** | I | II | III | P-value, ANOVA | I | II | III | P-value ANOVA |
| **Incidence (%)** | 61 | 80 | 77 | 0.18 | 52[a] | 80[b] | 77[b] | 0.027 |
| **Mean latency (weeks post-MNU injection)** | 17.9±1.1 | 16.5±0.9 | 16.2±0.9 | 0.40 | 17.6±1.2 | 16.5±0.9 | 16.2±0.9 | 0.53 |
| **Mean multiplicity** | 1.9±0.3 | 2.9±0.4 | 2.7±0.5 | 0.13 | 1.8±0.3 | 2.8±0.4 | 3.2±0.7 | 0.18 |
| **Mean volume ($cm^3$)** | 0.9±0.3[a] | 3.0±0.6[b] | 2.9±0.6[b] | 0.002 | 1.0±0.4[a] | 3.0±0.7[b] | 2.9±0.6[b] | 0.01 |
| **Mean weight (g)** | 0.5±0.1[a] | 1.1±0.2[a,b] | 1.3±0.2[b] | 0.019 | 0.6±0.2[a] | 1.1±0.3[a,b] | 1.3±0.2[b] | 0.069 |

1. Results are expressed as mean ± SEM. Means in a row with different letters at each time point significantly differ at $P<0.04$ by between-group comparisons.
2. Groups I, II and III received the 2 mg folic acid/kg diet (control) from weaning at 3 weeks of age for 5 weeks until one week following MNU injection (at 50 days of age), followed by the 0 (deficient), 2 (control) and 8 (supplemented) mg folic acid/kg diet, respectively, for 22 weeks until the time of sacrifice (30 weeks of age).

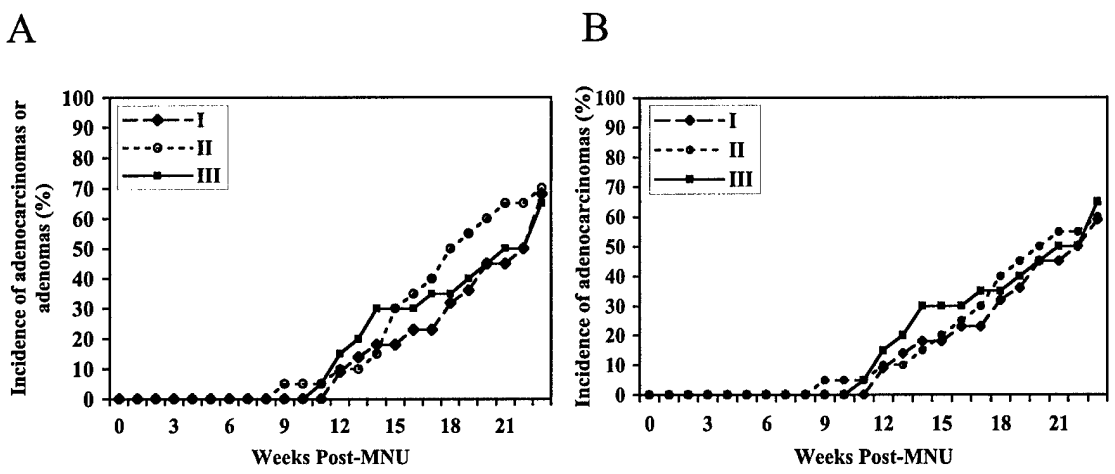
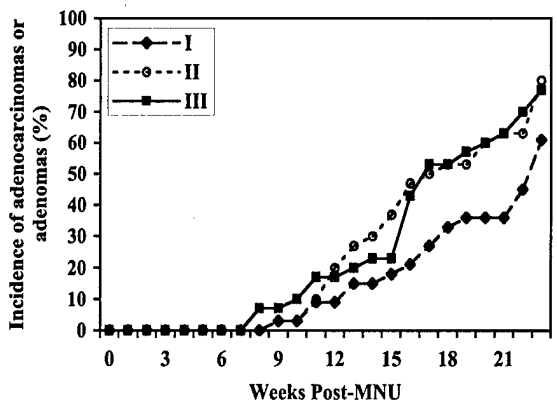

A
B
Incidence of adenocarcinomas or adenomas (%)
Incidence of adenocarcinomas (%)
I
II
III
0
10
20
30
40
50
60
70
80
90
100
0
3
6
9
12
15
18
21
Weeks Post-MNU
Weeks Post-MNU

Figure 1

A


Incidence of adenocarcinomas or adenomas (%)
100
90
80
70
60
50
40
30
20
10
0
I
II
III
0
3
6
9
12
15
18
21
Weeks Post-MNU

B

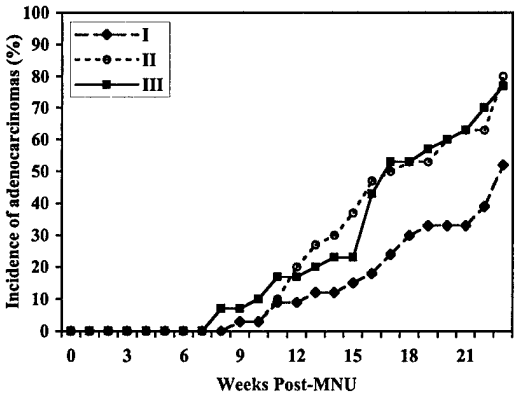

Incidence of adenocarcinomas (%)
100
90
80
70
60
50
40
30
20
10
0
I
II
III
0
3
6
9
12
15
18
21
Weeks Post-MNU

Figure 2

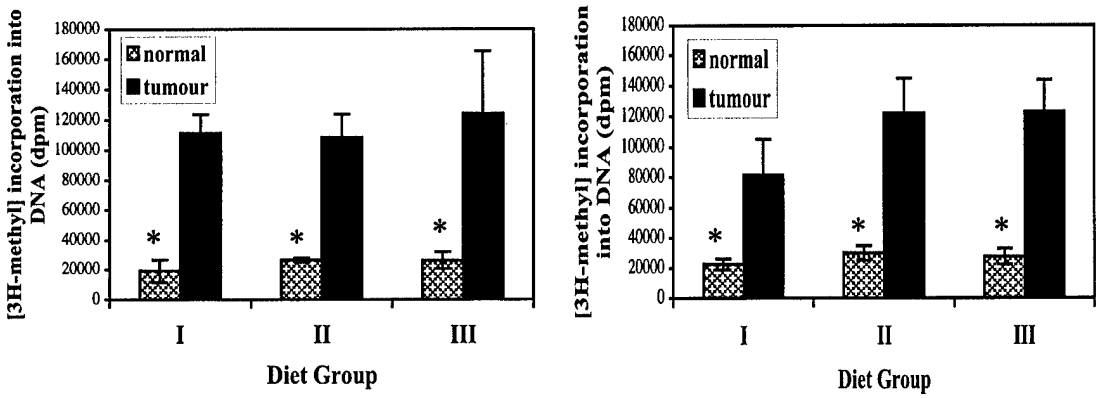

[3H-methyl] incorporation into DNA (dpm)
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
normal
tumour
*
I
II
III
Diet Group
[3H-methyl] incorporation into DNA (dpm)
normal
tumour
Diet Group

Figure 3